DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NOVEL β-ASPARTYL-AMP ANALOGS

By

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To My Family & Iwona

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TABLE OF CONTENTS

PAGE

ACKNOWLEDGMENTS iii

TABLE OF CONTENTS v

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT xi

CHAPTER

1 INTRODUCTION 1

- Asparagine Synthetase 1
- Chemotherapy for Acute Lymphoblastic Leukemia 4
- Inhibitors of Asparagine Synthetase B 5
- Human Asparagine Synthetase 11
- Summary 11
- Specific Aims 12

2 INHIBITOR DESIGN AND SYNTHETIC METHODOLOGY 13

- Modifications of the Phosphoramidate Linker 13
- Modifications of the Adenine and Ribose Moieties 17
- Modifications of the Aspartate Moiety 24

3 DESIGN AND SYNTHESIS 27

- Design of βAspAMP Analogs 27
- Synthetic Methodology 28
    - Synthesis of Sulfamoyl Adenosine and its Analogs. 28
    - Synthesis of Aspartate Analogs. 30
- Results and Discussion 31
    - Synthesis of Components. 31
    - Synthesis of *N*-acyladenosinesulfamates. 35
    - Synthesis of *N*-aspartateadenosinesulfamide. 41

Synthesis of *N*-acyladenosinesulfamates with Pyrrolidine-Based Aspartate Analog.......... 46
Synthesis of βAspAMP Analogs with Modified Adenosine Moiety.......... 58
Conclusion.......... 62

4 BIOLOGICAL EVALUATION OF SYNTHESIZED ANALOGS.......... 64

Specific Aims.......... 64
Kinetic Assays.......... 66
Results and Discussion.......... 67
Inhibition Analysis of Sulfamate **55**.......... 67
Inhibition Analysis of Sulfoximine **5**.......... 70

5 CONCLUSION AND FUTURE WORK.......... 73

6 EXPERIMENTAL SECTION.......... 76

Kinetic Assays.......... 76
Compound Characterization.......... 76

APPENDIX

A ENZYME INHIBITION GRAPHS.......... 112

B ^{1}H NMR DATA OF FINAL PRODUCTS.......... 121

REFERENCES.......... 138

BIOGRAPHICAL SKETCH.......... 146

LIST OF TABLES

TABLE PAGE

2-1. SAR of series of IleRS inhibitors 23
3-1. Deprotection attempts of **52**, **53** and **54** using hydrogenolysis 38
3-2. Deprotection attempts of **52**, **53** and **54** using catalytic transfer hydrogenation 39
3-3. Deprotection attempts of **52**, **53** and **54** using other methods 39
3-4. Attempts of deprotection of **72** 49

LIST OF FIGURES

FIGURE PAGE

1-1. Asparagine synthetase 2
1-2. Ammonia channel in AS........ 3
1-3. Catalytic mechanism of AS 4
1-4. Interesting inhibitors of AS-A and AS-B 6
1-5. Synthesis of phosphonate analog **2**........ 7
1-6. Synthesis of phosphoramidate **4** 8
1-7. Phosphoramidate **4** and sulfoximine **5**........ 9
1-8. Synthesis of sulfoximine **5** 10
2-1. Structures of sulfamoyl adenosine analogs 13
2-2. Some of the aminoacyl-tRNA synthetase inhibitors 14
2-3. AZT-triphosphate analog........ 15
2-4. Nucleocidin and 4'-fluorinated analog 15
2-5. Serine hydroxamate-AMP and 5'-*O*-[*N*-(L-seryl-sulfamoyl)] adenosine 16
2-6. Displacement of phosphoester with ester, amide etc........ 17
2-7. Structures of inhibitors with modified adenosine moiety........ 18
2-8. Comparison of structure to activity relation of vanilloid and isovanilloid analogs... 18
2-9. Tetrazole derivatives of aminoacyl adenylate as inhibitors........ 20
2-10. Displacement of adenosine with thiazole derivatives........ 21
2-11. Glutamyl-tRNA synthetase inhibitors 21
2-12. Example of design of more complex types of IleRS inhibitors........ 22
2-13. Nitroaniline-*N*-alcohols as non-nucleoside adenosine analogs 24
2-14. β-Alanine and succinic acid as aspartate analogs........ 25
2-15. Conformation of enzyme-bound aspartate and its analog 25
2-16. Synthesized aspartate analogs based on pyrrolidine structure........ 26
3-1. Strategy for the design of βAspAMP analogs 27
3-2. Synthesis of sylfamoyl adenosine derivatives........ 29
3-3. Synthesis of sylfamoyl adenosine derivatives using BTO 29
3-4. Synthesis of nitroaniline analogs of sulfamoyl adenosine........ 30
3-5. Aspartate analogs........ 31
3-6. Synthesis of protected adenosine sulfamate 33
3-7. Synthesis of aspartate analog **38**........ 33
3-8. Separation of isomeric benzyl esters 34
3-9. Synthesis of aspartate analogs **39** and **40** 34
3-10. Synthesis of fully protected intermediates **49**, **50** and **51**........ 35
3-11. Deprotection of acetonide; formation of **52**, **53** and **54**........ 36
3-12 Deprotection attempts of **52**, **53** and **54**........ 38
3-13. Summary of **55**, **56** and **57** synthesis........ 40

3-14. Synthesis of intermediate 2,3-*O*-propylidene adenosinesulfamide 41
3-15. Attempted coupling of **61** and **38a** 42
3-16. Synthesis of *N*-succinimide ester **38c** 43
3-17. Synthesis of fully protected intermediate **62** 43
3-18. Synthesis of **64** 44
3-19. Complete synthesis of sulfamide **65** 45
3-20. Aspartate analogs **67a** and **67b** 46
3-21. Synthesis of aspartate analog **69** 47
3-22. Oxidative double bond cleavage in **69** 48
3-23. Reduction of **71** 48
3-24 Deprotection attampts of **72** 49
3-25. Deprotection of **72** 50
3-26. ^{1}H NMR determination of the diastereomeric mixture ratio 51
3-27. Synthesis of **74a** and **74b** 52
3-28. Final step in synthesis of **76** 52
3-29. Synthesis of **78** 53
3-30. Oxidative cleavage of **78** 54
3-31. Synthesis of **82** 55
3-32. ^{1}H NMR analysis of **82** configuration 55
3-33. Full deprotection of **82** 56
3-34. Synthesis of **85** 57
3-35. Deprotection of **75b** and **84b** 58
3-36. Synthesis of sulfamates **90**, **91**, **92** and **93** 58
3-37. Synthesis of intermediates **94-102** 59
3-38. Final deprotection of **96**, **99** and **102** 60
3-39. Deprotection of **95**, **98** and **101** 61
3-40. Synthesis of **113-116** 62
4-1. Synthesized βAspAMP analogs 65
4-2. Progress curves for 0, 5, 10, 25 and 50 μM **55** at 0.5 mM ATP 67
4-3. Progress curves for 0, 5, 10, 25 and 50 μM **55** at 5 mM ATP 69
4-4. Plot of *k* versus [**55**] at 0.5 mM ATP 69
4-5. Progress curves for 0.5, 1, 2, 4, 6, and 10 μM **5** at 0.5 mM ATP 71
4-6. Progress curves for 0.5, 1, 2, 4, 6, and 10 μM **5** at 5 mM ATP 71
4-7. Plot of *k* versus [**5**] at 0.5 mM ATP 72
5-1. Structures of some novel βAspAMP analogs. 75
6-1. Chromatogram of **75a** and **75b** mixture 89
6-2. Chromatogram of **84a** and **84b** mixture 91
A-1. Inhibition graph of **55**. 112
A-2. Inhibition graph of **56**. 113
A-3. Inhibition graph of **57**. 113
A-4. Inhibition graph of **65**. 114
A-5. Inhibition graph of **76**. 114
A-6. Inhibition graph of **85**. 115
A-7. Inhibition graph of **103**. 115
A-8. Inhibition graph of **104**. 116
A-9. Inhibition graph of **105**. 116

A-10. Inhibition graph of **106.** 117
A-11. Inhibition graph of **107**. 117
A-12. Inhibition graph of **108**. 118
A-13. Inhibition graph of **113**. 118
A-14. Inhibition graph of **114**. 119
A-15. Inhibition graph of **115**. 119
A-16. Inhibition graph of **116**. 120

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DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NOVEL β-ASPARTYL-AMP ANALOGS

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Chair: Nigel G. J. Richards
Major Department: Chemistry

Human asparagine synthetase (hAS), a glutamine-dependent amidotransferase, catalyzes the synthesis of asparagine from aspartic acid, glutamine, and ATP. The catalytic mechanism of human asparagine synthetase is not fully understood. The X-ray structure of hAS in complex with substrates, intermediate, or inhibitors is not available.

Asparagine synthetase catalyzes the formation of the β-Aspartyl-AMP intermediate in one of the active sites. Stable analogs of acyladenylate intermediates are useful probes of the enzyme mechanism, and have a potential as enzyme inhibitors. It is believed that hAS inhibitors might find an application in cancer chemotherapy since asparagine depletion caused by administration of L-asparaginase is a currently implemented protocol for the treatment of acute lymphoblastic leukemia.

This study reports the design, synthesis and biological evaluation of a library of β-aspartyl-AMP analogs. A total of 16 analogs with different structural modifications have been prepared and fully characterized. All synthesized analogs were screened for inhibition activity toward hAS. One of the described compounds exhibits inhibition of human asparagine synthetase, with slow-onset kinetics, and measured K_i^* of 280 nM. These observations provide the basis for the discovery of new compounds with application in the treatment of drug-resistant leukemia.

CHAPTER 1
INTRODUCTION

Asparagine Synthetase

Asparagine synthetase (AS), an enzyme present in both prokaryotes and eukaryotes, catalyzes the conversion of aspartic acid to asparagine. This ATP-dependent reaction employs ammonia or glutamine as a nitrogen source.[1] So far, two different families of AS enzymes[2] that do not display any sequence similarity have been discovered in prokaryotes and eukaryotes. Strictly ammonia-dependent asparagine synthetases A (AS-A) is a family that appears to be evolutionarily related to amino-acyl tRNA synthetases.[3] The second class of AS, asparagine synthetases B (AS-B), prefer glutamine as a source of nitrogen (although these enzymes can also employ ammonia as an alternate substrate). Glutamine-dependent AS has been cloned and/or isolated from yeast,[4] plants,[5,6] bacteria,[7,8,9] and mammalian sources.[10,11,12] Sequence analyses have shown that these enzymes belong to the Class II (formerly purF) glutamine amidotransferase[13] (GAT) superfamily,[14] which also includes glutamine 5'-phosphoribosyl-1-pyrophosphate amidotransferase (GPA),[15] glutamine fructose-6-phosphate amidotransferase (GFAT),[16] and glutamate synthetase.[17] In humans, the only asparagine synthetase known is the glutamine-dependent form.

Even though the kinetic and chemical mechanisms of ammonia-dependent AS (such as *E. coli* AS-A) appears to be well understood,[18,19,20] full understanding of AS-B mechanism is still under investigation. A key step forward in studying the structure[21] and mechanism[22] of glutamine-dependent AS was development (in our laboratory) of the procedures for obtaining large amounts of AS-B, encoded by the *asnB* gene in *Escherichia coli*.[23,24] Successful determination of the three-dimensional structure of the ternary complex among glutamine, AMP, and the C1A asparagine synthetase B mutant was later accomplished.[21]

Asparagine synthetase B (AS-B) in its active form exists as a homodimer, where each of the monomers incorporates two domains. The N-terminal glutamine-binding domain (yellow in Figure 1-1) forms the glutaminase active site, and an asparagine-binding C-terminal domain (blue in Figure 1-1) forms the synthetase active site (Figure 1-1).

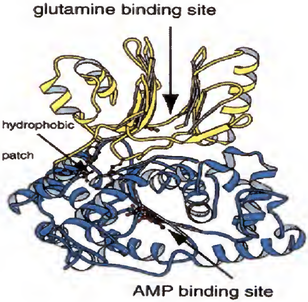


Figure 1-1. Asparagine synthetase

Asparagine synthetase B (AS-B) contains 553 amino acids. In its tertiary structure, residues 1-194 form the glutamine-binding domain; the asparagine-binding domain is formed by amino acids 195-516. Separated by approximately 19 Å, the two active sites are connected by a molecular tunnel,[21] in which the ammonia molecule is transported from one active site to another (Figure 1-2; the boundaries of the molecular tunnel are shown in red).

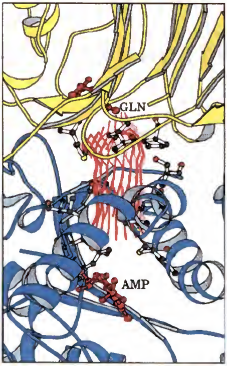


Figure 1-2. Ammonia channel in AS

According to presently available experimental data,[22] the overall transformation of aspartic acid to asparagine occurs via three separate reactions (Figure 1-3). In the glutaminase site, glutamine is hydrolyzed to glutamate and ammonia; which is then transported via the molecular tunnel to the synthetase active site, where aspartate is activated by reaction with ATP to form the β-aspartyl-AMP intermediate **1** (βAspAMP) with release of pyrophosphate. Breakdown of **1** with release of AMP occurs through nucleophilic attack of ammonia produced in the hydrolysis of glutamine.

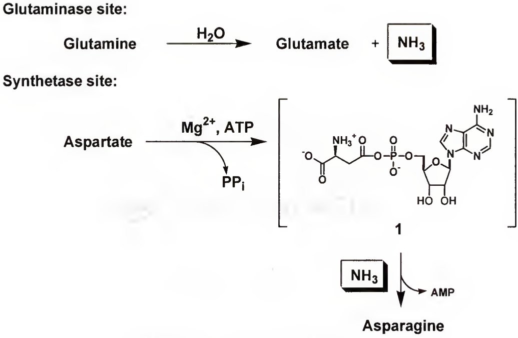


Figure 1-3. Catalytic mechanism of AS

Despite this progress, some of the key structure and mechanistic enzymology issues remain unknown. The structural organization of the active site involved in activation of aspartate is still unknown. Recent X-ray structure of the *E. coli* AS-B mutant complex displays some structural distortions in the synthetase domain caused by the absence of aspartate. Also the molecular basis for the coordination of two half-reactions that occur during aspartate synthesis remain unidentified. The basis for the ammonia channel also needs to be investigated.

Chemotherapy for Acute Lymphoblastic Leukemia

Use of L-asparaginase,[25,26,27] (the enzyme responsible for hydrolysis of asparagine to aspartate) is a common chemotherapeutic protocol in the treatment of acute lymphoblastic leukemia (ALL)[28,29] and acute myeloblactic leukemia.[30,31] This therapeutic approach produces a variety of side effects.[32] Moreover, large numbers of patients who achieve remission suffer a relapse with tumors that are resistant to further L-asparaginase therapy.[31,32] Furthermore, L-asparaginase increases the growth of resistant tumors, and increases their metastatic activity.[34] There is no reliable protocol by which the sensitivity of a particular type of tumor to L-asparaginase therapy can be predicted; therefore, the major use of this enzyme remains confined to ALL. Regardless, estimates suggest that 5-10% of solid tumors, including those that appear to be smoking-related, may be sensitive to asparagine depletion.[33]

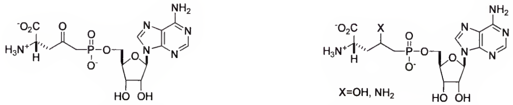
Inhibitors of Asparagine Synthetase B

Asparagine synthetase is a potential target for cancer chemotherapy, since asparagine depletion caused by administration of L-asparaginase is a currently implemented protocol for the treatment of acute lymphoblastic leukemia.[34] Unfortunately, in many cases the use of L-asparaginase is limited by formation of tumors resistant to this type of therapy. Recently, Aslanian *et al.* [35] reported that expression of AS alone is sufficient to induce L-asparaginase resistance in leukemia cells. Based on these results, we can envision potent and specific AS-B inhibitors as being potentially valuable agents for treating leukemia, and as tools in investigating the cellular basis of L-asparaginase resistance.[2] Furthermore, the recognition that T-cell-based immunosupression is often observed in patients undergoing initial treatment with L-asparaginase provides an additional incentive to find AS inhibitors.

Although large-scale screening of analogs of glutamine, ATP and aspartic acid did reveal compounds that have some inhibitory activity in either *in vivo* or *in vitro* study, their specificity and/or potency was marginal.

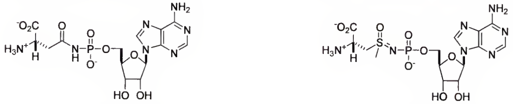
From a theoretical standpoint, our recent X-ray structure of AS-B did not include the active site of the synthetase domain. Additional evidence is also required to confirm the existence of an ammonia channel. Hence, an inhibitor that is highly efficient against a particular catalytic domain is highly desirable, since it would provide valuable information on enzyme structure, on interactions that catalyze the formation and breakdown of the βAspAMP intermediate, and on the molecular mechanism of channel formation. Inhibitors designed in this approach should be able to mimic βAspAMP, based on the fact that intermediate and transition-state analogs bind tightly to enzyme active site.

So far, only four inhibitors[36,37,38,39] have been reported that mimic βAspAMP, with three of them displaying very potent activity (Figure 1-4).



2
K_i: 80 μM (AS-A from Mung beans)

3
K_i: 100 nM (AS-A from white lupine or *E. coli*)

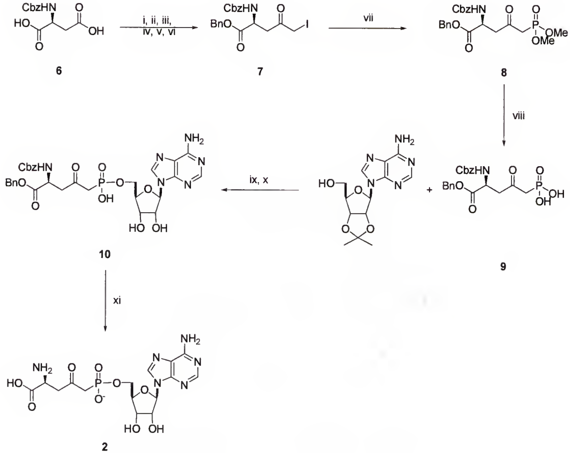


4
IC_{50}: 200 nM (AS-B from *E. coli*)

5
K_i: 67 nM (AS-A from *E. coli*)

Figure 1-4. Interesting inhibitors of AS-A and AS-B

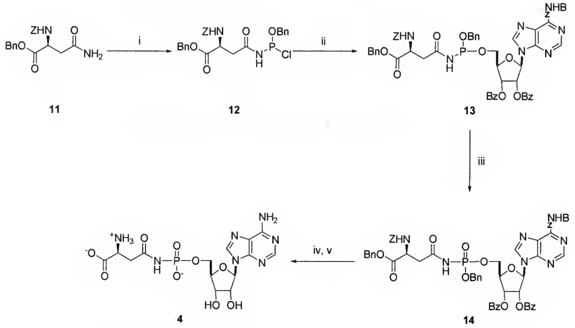
Phosphonate **2** [36] has shown moderate inhibition properties toward AS-A isolated from Mung beans. A major part of its 11-step synthesis (Figure 1-5) focuses on conversion of aspartate analog **6** into phosphonopentanoic acid analog **9** which can be used for further coupling. Even though the initial inhibition study yielded promising results (K_i of 80 μM), the published synthesis is not suitable for obtaining further analogs (mainly because of problematic synthesis of the modified amino acid moieties). Similar problems arise in the published, analogous synthesis of phosphonate **3.**[37]



i. $(AcO)_2O$; ii. BnOH; iii. $SOCl_2$; iv. CH_2N_2; v. HCl; vi NaI, Acetone; vii. $P(OCH_3)_3$, reflux; viii. TMSI, MeCN ; ix. DCC, Pyridine; x. 10% AcOH, reflux; xi. H_2, 10% Pd/C

Figure 1-5. Synthesis of phosphonate analog **2**

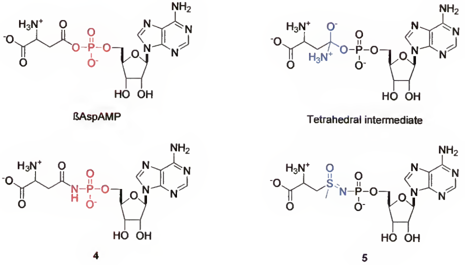
Phosphoramidate **4**[39] was synthesized in our labs (Figure 1-6) using a novel "one-pot" protocol for the coupling of **12** with *N*-benzoyl-2',3'-di-*O*-benzoyladenosine. This synthesis is designed in such a way that an approach to analogs with different amino acid moieties should be limited only by availability of different amides, such as **11**. The main drawbacks of this procedure included poor yield of the coupling reaction, and difficult separation of product from formed byproducts. Additionally, the stability of the phosphoramide bond creates another disadvantage that needs to be overcome in the approach to clinically useful, stable inhibitors.



i. $BnOPCl_2$, $(i\text{Pr})_2NEt$, CH_2Cl_2; ii. $(i\text{Pr})_2Net$, CH_2Cl_2, *N*-benzoyl-2',3'-di-*O*-benzoyladenosine; iii. *t*BuOOH, CH_2Cl_2; iv. 10% Pd/C, 1,4-cyclohexadiene; v. NH_4OH

Figure 1-6. Synthesis of phosphoramidate **4**

A distinctively different approach is displayed in Koizumi's[38] synthesis of sulfoximine **5**. Previous inhibitors **2**, **3** and **4** were designed and synthesized to mimic βAspAMP; therefore they probably act as (the intermediate) analogs in binding to the AS synthetase site. In reaction catalyzed by AS, asparagine is formed by nuclephilic attack of ammonia on βAspAMP. Therefore, the apparently formed transition state must have the structure and properties of a tetrahedral intermediate formed in reactions of nuclephiles with carbonyl moiety. The tetrahedral intermediate is characterized by negative charge localized on the oxygen atom and positive charge on the heteroatom (i.e. ammonia in the AS catalyzed reaction).



ßAspAMP

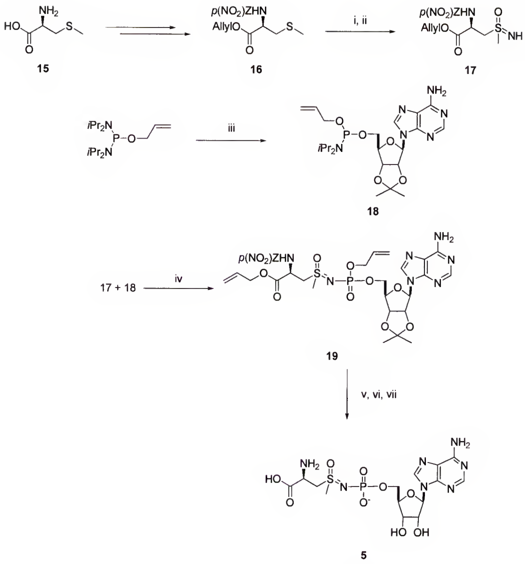
Tetrahedral intermediate

4

5

Figure 1-7. Phosphoramidate **4** and sulfoximine **5**

Introduction of the sulfoximine moiety in compound **5** creates a very clever transition state analog. In comparison of **5** to tetrahedral intermediate formed from βAspAMP we can envision that the methyl group in sulfoximine bears a partial positive charge caused by electronegative atoms connected to sulfur (oxygen and nitrogen). In that way the methyl group mimics positively charged ammonia in transition state. Additionally, the partial negative charge in **5** is spread among oxygen and nitrogen, again mimicking the transition state (Figure 1-7). Koizumi's synthesis[38] (Figure 1-8) offers a quick and elegant approach to a stable and potent inhibitor.



i. $NaIO_4$, $MeOH/H_2O$; ii. *O*-mesitylsulfonylhydroxylamine, MeCN;

iii. 2',3'-*O*-isopropylideneadenosine, diisopropylammonium 1*H*-tetrazolide, Py;

iv. 1*H*-tetrazole, MeCN, *t*BuOOH; v. TFA/H_2O; vi. $Pd(PPh_3)_4$, PPh_3, HCOOH, NEt_3, THF,

vii. H_2, Pd/C, $MeCN/H_2O$).

Figure 1-8. Synthesis of sulfoximine **5**

A number of difficulties in obtaining libraries of analogs can be foreseen. Synthesis of analogs with different amino acid moieties is limited to availability of different methyl cysteines and the fact that one of the crucial steps involves use of a hazardous reagent.

Additionally, the synthesized sulfoximine is obtained as a mixture of diastereoisomers with respect to sulfur atom; therefore, the study on separate diastereoisomers is necessary.

Human Asparagine Synthetase

Since our main objective is to synthesize and characterize clinically useful AS inhibitors, it was crucial to gain access to large quantities of human enzyme.[40,41] This was accomplished by developing a baculovirus-based expression system in which the gene encoding human asparagine synthetase (hAS) was modified. As a result the expressed enzyme would contain additional residues at the C-terminus corresponding to a *c-myc* tag. The resulting construct was then cloned into a pBAC-1 transfer vector, which includes a C-terminal histidine tag for protein purification. That allows the control of the expression by the *polh* promoter and leads to high levels of protein expression during late phases of viral infection. Recombination of pBac-1 with theBacVector-3000 construct was then used to obtain a baculovirus capable of replicating and infecting conditioned *Sf9* cells, which also contained the gene encoding human AS. Human AS was then expressed using standard procedures in conditioned *Sf9* cells followed by purification of the obtained enzyme on a pre-equilibrated Ni-Agarose column.[42] This procedure resulted in multi-milligram amounts of wild type enzyme, which displayed ammonia-dependent synthetase activity similar to that reported for recombinant, human AS obtained using a yeast-based expression system.[43,44,45]

Summary

Only limited progress has been reported in the effort to design and synthesize inhibitors of asparagine synthetase. Published structures of βAspAMP analogs that act as AS inhibitors provide valuable insight into the recognition of the substrates and inhibitors by the enzyme. This has a great impact on further studies in this field. Additionally, we might be able to correlate inhibitors designed for similar enzymes to our efforts in this field. Aminoacyl-tRNA synthetases (aaRSs) catalyze reaction similar to that in the AS synthetase site, where an amino acid is activated to form aminoacyl adenylate with use of ATP and Mg^{2+} or Mn^{2+}. Widely published results on aaRS inhibitor design and synthesis might be valuable for our studies, for that reason the next chapter provides a short review of this subject.

Specific Aims

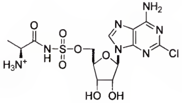
We are very interested in designing and studying new inhibitors of the human asparagine synthetase. We would like to design and synthesize a small library of novel βAspAMP analogs, potential inhibitors of hAS. Our desired approach would be suitable for parallel synthesis requirements (making modifications of different moieties within the structure much easier), therefore making the entire library more available and expandable. All synthesized compounds will be biologically evaluated for activity toward inhibition of hAS using standard, used in our laboratory assays.

Information obtained from synthesis as well as biological study will be very helpful for our design of new inhibitors. Additionally, we would like to employ molecular modeling methods to help us understand the structure and catalytic mechanism of hAS.

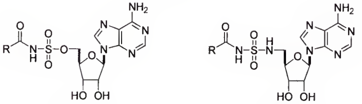
CHAPTER 2
INHIBITOR DESIGN AND SYNTHETIC METHODOLOGY

Modifications of the Phosphoramidate Linker

The unstable phosphatee moiety in βAspAMP was replaced by phoshoramidate in **4**, where the oxygen atom of the mixed anhydride bond is replaced by -NH. It is likely that the P-N bond of the N-acylphosphoramidate is much more stable under physiological conditions.



Acsamycin

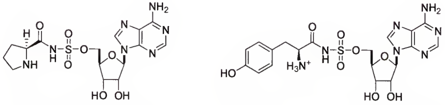


Sulfamate

Sulfamide

Figure 2-1. Structures of sulfamoyl adenosine analogs

It has recently been demonstrated by using crystal structures of seryl-tRNA synthetase[46] that sulfamoyl moieties in either sulfamate or sulfamide form (Figure 2-1) can substitute for a phosphate in certain enzyme systems. Amino acid sulfamoyl adenosine moieties of this type are found in natural products such as ascamycin, a nucleoside antibiotic from *Streptomyces* (Figure 2-1).[47] A number of known aminoacyl sulfamates act as potent inhibitors of a number of enzymes including aminoacyl-tRNA synthetases (aaRSs), enzymes responsible for catalyzing reactions similar to those of hAS, in which an amino acid is activated to form aminoacyl adenylate with use of ATP and Mg^{2+} or Mn^{2+}. Some of the examples are presented in Figure 2-2.



K_i = 0.6 nM (human ProRS)

IC_{50} = 26 nM (*Staphylococcus aureus* ArgRS)

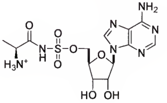
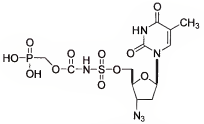


Figure 2-2. Some of the aminoacyl-tRNA synthetase inhibitors

Arginyl sulfamate inhibits the *Staphylococcus aureus* ArgRS with an IC_{50} of 4.5 nM;[48] threonyl sulfamate with an IC_{50} of 15 nM;[48] histydyl sulfamate with an IC_{50} of 130 nM;[48] isoleucyl sulfamate with an IC_{50} of 4 nM;[49] tyrosyl sulfamate with an IC_{50} of 26 nM;[50] glutaminyl sulfamate inhibits GlnRS with a K_i of 1.32 μM;[51] prolyl sulfamate inhibits the human ProRS with a K_i of 0.6 nM and E. coli ProRS with a K_i of 4.3 nM.[52]

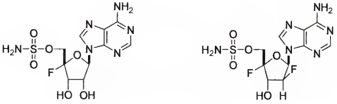
An analogous approach was used by Vince *et al.*[53] in a synthesis of an analog of AZT-triphosphate, which is a membrane permeable HIV reverse transcriptase inhibitor. A sulfamoyl moiety was used as a replacement for the α-phosphate (Figure 2-3). Even though the inhibition studies on recombinant HIV-1 reverse transcriptase revealed the IC_{50} for the sulfamoyl analog was 300 μM (1 μM for the AZT-triphosphate), it was shown that the modified sulfamoyl group phosphate does exhibit binding recognition.

A number of different compounds with the sulfamoyl group, although lacking the acyl moiety, have also been reported in the literature. The majority of them display interesting biological activity (Figure 2-4).



AZT-triphosphate analog

Figure 2-3. AZT-triphosphate analog



Nucleocidin

4'-fuorinated Nucleocidin

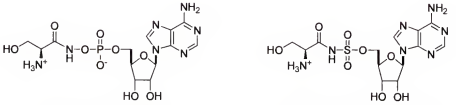
Figure 2-4. Nucleocidin and 4'-fluorinated analog

Nucleocidin, natural product isolated form *Streptomyces calvus* displays antitrypansomal antibiotic activity.[54] Furthermore, a group of Glaxo scientists reported[55] that the 4'-fluorinated analog displays some potent antiviral activity, in their case against

Herpes simplex viruses. Both compounds are currently being investigated for potential activity against HIV.

5'-N-sulfamoyl adenosine, in which the phoshoramidate bond between the amino acid and adenosine moiety has been replaced with a sulfamide is another type of nonhydrolyzable sulfamoyl analog. A series of such compounds have been recently reviewed.[56]

Amino acid hydroxamates are known to act as analogs of the amino acid in biological systems. Hence, the amino acid-hydroxamate-ATP might as well act as the analog of the aminoacyl adenylate. It has been observed that serine hydroxamate-AMP binds very similarly with seryl-tRNA synthetase as 5'-*O*-[*N*-(L-seryl-sulfamoyl)] adenosine (Figure 2-5).[46]

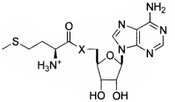


Seryl-hydroxamate-AMP

5'-O-(*N*-seryl-sulfamoyl)adenosine

Figure 2-5. Serine hydroxamate-AMP and 5'-*O*-[*N*-(L-seryl-sulfamoyl)] adenosine

One of the simpler replacements of the labile acylphosphate linkage are ester and amide bonds (Figure 2-6).[57] Ester **20** and amide **21** analogs of methionyl adenylate displayed in Figure 2-6 were shown to be strong inhibitors of methionyl-tRNA synthetase (metRS) from *E. coli*, *Mycobacterium tuberculosis*, *Saccharomyces cereisiae* and humans.



20. X=O
21. X=NH
22. X=N-OH
23. X=NH-O

Figure 2-6. Displacement of phosphoester with ester, amide etc

Similarly to L-methionine hydroxamate being a good inhibitor of MetRS, N-alkyl hydroxamate analog **22** and O-alkyl hydroxamate analog **23** were shown to have good activity. Ester analog **20** was found to be the most potent against *E. coli* MetRS in this series, and the relative activities are in following order: **20** (Ester) > **22** (hydroxamate) > **21** (amide) > **23** (O-hydroxamate) = hydroxamate. The estimated K_i of **20** and **22** were 10.9 μM and 13.1 μM respectively.

Modifications of the Adenine and Ribose Moieties

Recognition of the adenine ring is very important for the inhibitor binding. Consequently, designing analogs for this structural moiety is crucial for the development of potent inhibitors. A variety of different approaches to this problem have been reported.[56-66]

Replacing adenine of glutamoyl adenylate by other bases (purine, cytosine, dihydrocytosine, uridine) results in a greater that 100-fold loss in potency.[58] Introduction of different linkers between the 1'-position of ribose and adenine surrogates has been reported. Methionyl-tRNA and isoleucyl-tRNA synthetase inhibitors have been investigated (Figure 2-7).[57] Ester analog **24** has been described as a potent inhibitor of *E. coli* methionyl-tRNA synthetase (IC_{50} of 3.6 μM).

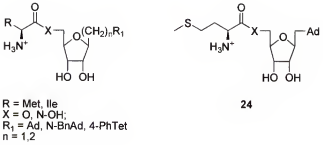


Figure 2-7. Structures of inhibitors with modified adenosine moiety

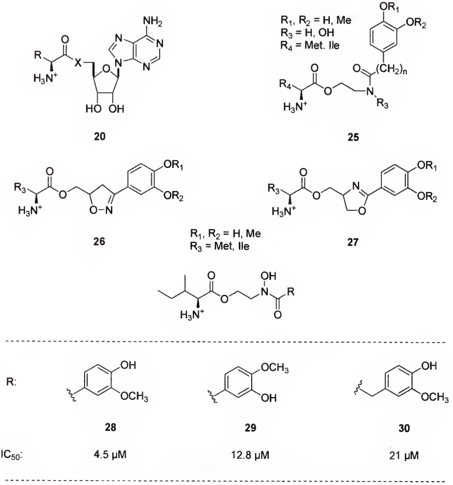


Figure 2-8. Comparison of structure to activity relation of vanilloid and isovanilloid analogs as inhibitors of methionyl-tRNA and isoleucyl-tRNA synthetases

An ester analog of acyl adenylate **20** was used as a lead compound and the adenine and ribose moieties were modified with corresponding bioisosters (Figure 2-8).[59] Vanilloid and isovanilloid moieties were used to replace adenine. The phenolic hydroxyl and methoxy groups were supposed to mimic the 6-NH_2 of adenine as a hydrogen donor, and the N-1 of adenine as a hydrogen acceptor, respectively. Simultaneously, ribose was replaced by its biosters, such as acyclic amide and hydroxamate in **25**, dihydroisooxazole in **26** and dihydrooxazole in **27**. Incorporation of those ribose mimics was based on published evidence describing them as useful, pharmacologically active adenylates in drug design.[60] The isovanillic hydroxamate **28** was found to be the most potent against IleRS. Most of the designed this way MetRS inhibitors displayed moderate activity against *E. coli* enzyme (IC_{50} = 32.5-163.5 μM).

Another commonly used way of modification of the adenine part of the aminoacyl adenylate is the use of different heterocycles. A number of synthesized novel tetrazole derivatives were found to display potent inhibition activity toward isoleucyl-tRNA synthetase from *E. coli* (Figure 2-9).[61]

Replacement of the adenine ring with a variety of other heterocycles, such as thiazole derivatives, has also been investigated (Figure 2-10).[62] Isoleucine phenyl thiazole analog **31** inhibits IleRS with an IC_{50} of 0.1 μM. It has been established that compound **31** acts as a competitive inhibitor with respect to isoleucine and ATP. Alanine analog **32** inhibits AlaRS respectively with an IC_{50} of 0.5 μM. Introduction of a methoxy group on the phenyl ring or replacement of the phenyl group with furan ring or alkenyl phenyl group increases the inhibition properties.

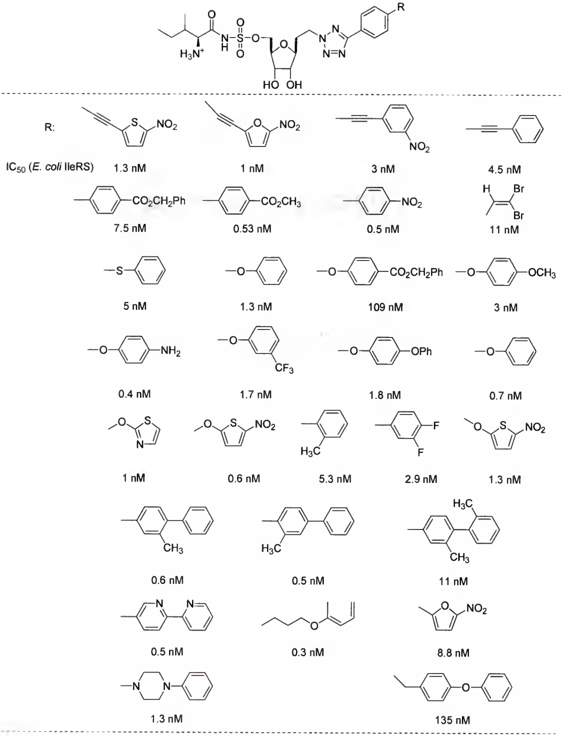
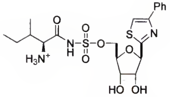


Figure 2-9. Tetrazole derivatives of aminoacyl adenylate as inhibitors of isoleucyl-tRNA synthetase



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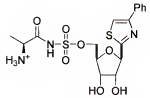

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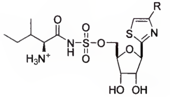
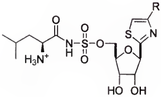


33

R: 4'-cyano-furanyl, 2'-OMe-Ph, 3'-OMe-Ph, PhOPh, 4'-OMe-Ph, 6'-OMe-2-naphtyl, CH_2CH_2Ph

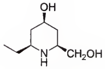


34

R: PhOPh, CH_2CH_2Ph, CH_2CH_2PhOPh

Figure 2-10. Displacement of adenosine with thiazole derivatives

One potential problem in this approach is associated with the low selectivity of bacterial aaRS when compared to human enzymes. For example leucine analogs **34** demonstrate high selectivity for *E. coli* LeuRS (2-16 nM) versus the human enzyme (1 μM).



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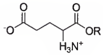

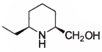

36

Figure 2-11. Glutamyl-tRNA synthetase inhibitors

Glutamate esters[63] with ribose, prolinol or substituted piperidine as the alcohol moiety were synthesized and screened for inhibition activity toward glutamyl-tRNA synthetase from *E. coli* (Figure 2-11). Piperidine derivatives **35** and **36** displayed the best inhibition properties with K_i of 20 μM and 230 μM respectively.

Information on structure-activity relation (SAR) gathered from number of synthetase inhibitors proved to be important for the design and synthesis of novel, much more complex synthetase-specific drugs.[56] Natural product mupirocin acts as an analog of isoleucyl-adenylate (Ile-AMP). It blocks the binding site of Ile-AMP by binding to the catalytic domain of the *T. thermophilus* IleRS.[64] It has been established that the pyran ring and α,β-unsaturated ester moiety of mupirocin mimic the ribose and adenine moiety of Ile-AMP. Consequently, a drug design approach was developed in which these two structural motifs were retained, while the amino acid side chain and the linker were varied (Figure 2-12). A series of inhibitors was synthesized based on this approach and their binding activities were studied (Table 2-1).[65]

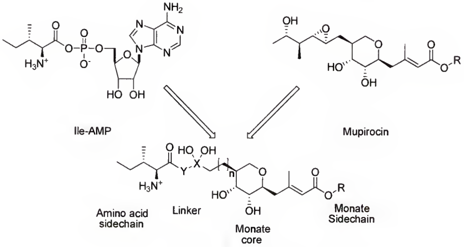

Figure 2-12. Example of design of more complex types of IleRS inhibitors

Table 2-1. SAR of series of IleRS inhibitors

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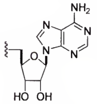

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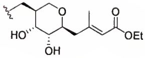

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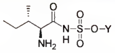
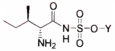
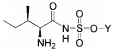
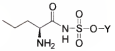
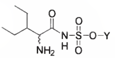
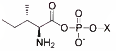
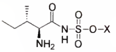
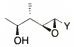
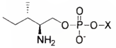
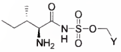
	<0.001
	0.7
	4.8
	0.007
	0.56
	50
	0.01
	0.19
	0.02
	1.4

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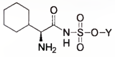
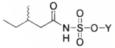
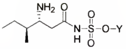
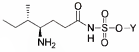
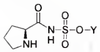
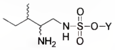
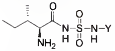
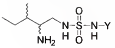
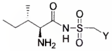
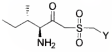
	0.55
	6900
	0.31
	55
	230
	2.0
	0.4
	900
	0.04
	0.6

Data obtained proves that the combination of the structural features of two different classes of inhibitors can be a base for an effective approach to improvement in potency.

Wang *et al.* [66] reported that different nitoaniline-*N*-alcohols, when phosphorylated might be used as a source of energy for different types of cells and enzymes (Figure 2-13).

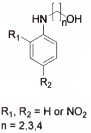


Figure 2-13. Nitroaniline-*N*-alcohols as non-nucleoside adenosine analogs

Based on this observation one can foresee similar compounds being good non-nucleoside mimics of adenosine. This particular approach has not been used in any published drug-design approach, although the simplicity of an approach to a number of different nitroaniline-N-alcohols makes it intriguing.

Modifications of the Aspartate Moiety

One can envision simple modifications of the aspartate moiety in an approach to the library of βAspAMP analogs (Figure 2-14). β-Alanine and succinate are simple analogs of aspartate, each lacking one of the groups, carboxyl and amino group respectively, presumably involved in hydrogen bonding interactions with the synthetase active site.

Recently Parr *et al.* [67] pursued a different approach. Three different examples of aspartate binding sites, for which the X-ray structures have been reported[68,69,70], display strong interactions between carboxylate groups of aspartate and arginine residues of the studied enzyme. It is highly feasible that the conformation of bound to the active site aspartate is such that it interacts with only one arginine residue (Figure 2-15).

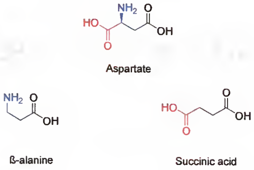


Figure 2-14. β-Alanine and succinic acid as aspartate analogs

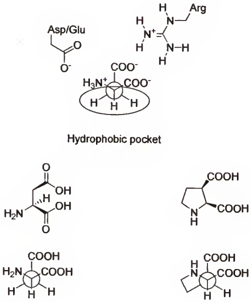


Figure 2-15. Conformation of enzyme-bound aspartate and its analog

Available data suggests that α and β protons of the aspartate are placed within well defined pocket on the surface of the enzyme, although there is enough space in this site to accommodate a small hydrophobic substituent. One can imagine using a pyrrolidine ring system to fix the conformation of aspartate. Designed in this way aspartate analogs were found to inhibit AS-B from *E. coli* with K_i in millimolar range (Figure 2-16).

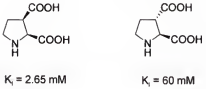


Figure 2-16. Synthesized aspartate analogs based on pyrrolidine structure

CHAPTER 3
DESIGN AND SYNTHESIS

Design of βAspAMP Analogs

Our goal was to construct a small library of βAspAMP analogs based on structure modifications of previously synthesized in our laboratory phoshoramidate **4**.[39] One can envision three distinct structural motifs in the structure of **4**: adenosine, phosphoramide linker and amino acid. By modifying each of these motifs we would be able to build the library in quick and effective fashion (Figure 3-1).

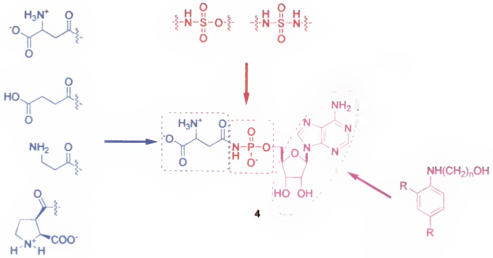


Figure 3-1. Strategy for the design of βAspAMP analogs

We used adenosine as well as novel, non-nucleoside analogs based on nitroanilline-*N*-alcohol.[66] The methodology for standard transformations in adenosine-based inhibitors is well established. Additionally, different nitroaniline analogs could be synthesized in effective fashion using standard transformations.

Aspartate and its β-alanine and succinate analogs allowed us to probe the active site of hAS for the importance of the interactions with α-carboxyl and β-amino groups. Additionally, use of pyrrolidine based aspartate[67] analogs provided us useful information on the possibility of accommodating additional groups in the active site.

We have chosen to replace the phosphoramidate moiety by a nearly isosteric, yet hydrolytically more stable, sulfamoyl link in either sulfamate (N-S-O) or sulfamide (N-S-N) form. This would not only make the target molecule more stable, but also would eliminate any charges associated with oxygen atoms in phosphoramidate linker. We would use the sulfamate over sulfamide for the first inhibitors because of simpler preparation due to the lack of requirement for use of an additional protecting group.

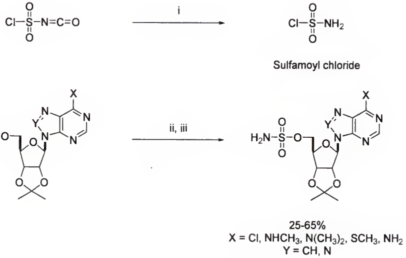
Synthetic Methodology

Synthesis of Sulfamoyl Adenosine and its Analogs.

Two different procedures are commonly used for conversion of alcohols into the corresponding sulfamoyl derivatives. Both methods use sulfamoyl chloride, prepared by acidic hydrolysis of chlorosulfonyl isocyanate[71] (Figure 3-2) as an elecrophilic source of the H_2NSO_2- moiety. Peterson *et al.*[71] used sodium hydride in DME to deprotonate the 5'- hydroxyl in the adenosine derivative and reacted the formed alkoxide with an excess of sulfamoyl chloride (Figure 3-2). The sulfamoylated derivatives were isolated in low to moderate yields. A similar method carried out in dioxane was reported by Shuman.[72]

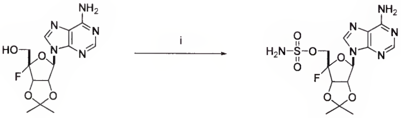
Jenkins *et al.*[73] reported some limitations of this method in the synthesis of nucleocidin. The use of sodium hydride yielded only trace amounts of acetonide-protected nucleocidin **37** (Figure 3-3). On the other hand, activation of 5'-hydroxyl with *bis*-tributyltin oxide (BTO) in refluxing benzene followed by reaction of the tributylstannylene formed with sulfamoyl chloride afforded the desired **37** in 87% yield.

For our purposes, both methods were evaluated and the most convenient was used for further synthesis.



i. HCl; ii. NaH, DME; iii. Sulfamoyl chloride

Figure 3-2. Synthesis of sylfamoyl adenosine derivatives

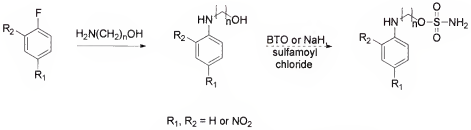


37

i. BTO, benzene, sulfamoyl chloride, reflux

Figure 3-3. Synthesis of sylfamoyl adenosine derivatives using BTO

Nitroaniline-*N*-alcohols are easily accessible by Sanger-type reaction[74,75] of fluoro(di)nitrobenzene with the corresponding amino alcohol (Figure 3-4). Alcohols obtained in this fashion were converted into the corresponding sulfamoyl derivatives using the previously described methodology.



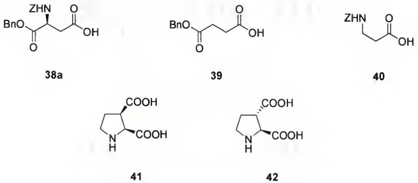
i. $H_2N(CH_2)_nOH$; ii. BTO or NaH, sulfamoyl chloride

Figure 3-4. Synthesis of nitroaniline analogs of sulfamoyl adenosine

Synthesis of Aspartate Analogs.

One of the key issues in the design and synthesis of a library of βAspAMP analogs is the availability of different aspartate analogs. One of the more challenging problems is the synthesis of these types of analogs with an appropriate pattern of protecting groups. It is crucial for the correct structure of the final product that the carboxyl protecting group be placed specifically on the α-carboxyl with the β-carboxyl unprotected, and therefore reactive in further coupling reaction with sulfamoyl adenosine and analogs. Hence, it is important to find or develop methods for preparation of aspartate analogs with correct protection pattern.

Aspartate analog **38a** can be prepared in two steps from commercially available *N*-carbobenzyloxy-L-aspartic acid;[76, 77] succinate **39** in one step from succinic anhydride;[78] substrate **40** in one step from β-alanine (Figure 3-5).[79,80,81] The synthesis of fully deprotected aspartate analogs **41** and **42** has been published,[67] although the strategy needed to be modified in order to obtain analogs with the correct protection pattern. It is also important to identify protecting groups suitable for our synthesis condition. Benzyl (Bn) and *N*-carbobenzyloxy (Z or Cbz) groups were chosen because of the ease of deprotection by hydrogenation, Lewis acid conditions or by use of a variety of strong nucleophiles.



38a **39** **40**

41 **42**

Figure 3-5. Aspartate analogs

Sulfamoyl adenosine and aspartate analogs obtained in this way were coupled using standard carbodiimide coupling conditions.[82,83,84] EDCI was used as a coupling reagent due to the ease of removal of formed in the reaction ureas.

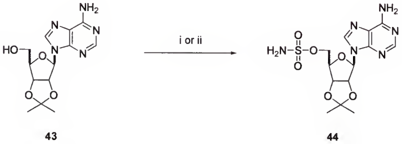
Results and Discussion

Synthesis of Components.

In attempts to find the most suitable method for conversion of commercially available 2,3-*O*-propylidene adenosine **43** into corresponding sulfamate **44,** we decided to test all published methods (Figure 3-6).[71,72,73] Compound **43** was refluxed in anhydrous benzene in the presence of 3 equivalents of bis(tributyltin)oxide (BTO) for 24 hours. Treatment of the formed tributylstannylene with 4 equivalents of sulfamoyl chloride resulted only in partial conversion (50%) to desired derivative. Similar conditions in dioxane gave only slightly improved yield of this transformation (55-60%). Moreover, the high molecular mass of used in excess BTO reagent and its high price, in combination with challenging workup and problems with complete removal prompted us to look elsewhere for a method suitable for large, multigram preparation of **44**. Our initial results employing 1.1 equivalent of sodium hydride in THF, followed by treatment with 4 equivalents of sulfamoyl chloride, indicated complete conversion to **44** (TLC). It was somewhat surprising to find that the final yield of this reaction was only in the 30-40% range. We suspected the aqueous workup to be responsible for partial decomposition and loss of a large part of our product. Therefore, we employed a 'no-workup' procedure for this reaction. The remaining sodium hydride was quenched with methanol, the solvent was removed and the residue absorbed on silica gel. Dry-load flash column chromatography yielded **44** in 95% isolated yield. The correct structure of **44** was confirmed by IR (1150 and 1350 cm^{-1} typical for H_2NSO_2O-) and shift of 5' CH_2- in the H^1 NMR compared to 2,3-*O*-propylidene adenosine. Spectral data was matched to those previously published.[50] This particular method allowed us to access large amounts of **44** suitable for further transformations.

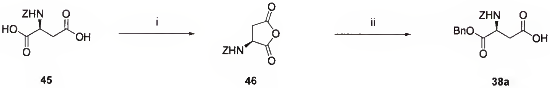
In the process of our research we found the purity of **44** to be crucial for further transformations. Therefore, in the following preparations, we tried to limit the amount of sulfamoyl chloride used in this transformation to 2-3 equivalents. It was observed that larger excesses of sulfamoyl chloride caused contamination of **44** even after column purification. Material contaminated in this way behaved poorly in further synthetic steps.

As previously mentioned, the availability and expandability of the constructed library was limited to accessibility of different aspartate analogs bearing an appropriate protection pattern. For synthesis of the correctly protected aspartate **38a** we employed previously published procedures (Figure 3-7).[76,77]



i. BTO, benzene (dioxane), sulfamoyl chloride; ii. NaH, THF, sulfamoyl chloride

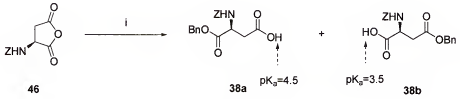
Figure 3-6. Synthesis of protected adenosine sulfamate



i. Ac_2O, RT; ii. BnOH, 80 °C.

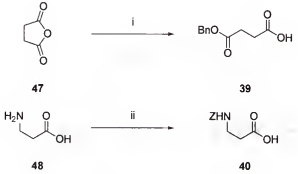
Figure 3-7. Synthesis of aspartate analog **38**

Commercially available *N*-carbobenzyloxy-L-aspartic acid **45** was converted to the corresponding anhydride **46** in moderate to high yield (75-90%) using the method of LeQuesne and Young.[76] Heating crude **46** in benzyl alcohol according to Bergmann, Zervas and Salzmann[77] resulted in opening of the anhydride and formation of two isomeric benzyl esters **38a** and **38b** in a 3:1 ratio respectively (Figure 3-8). Inseparable by standard chromatographic methods, **38a** and **38b** were isolated using fractional extraction based on the difference in the carboxylic acid pK_a. The structure and configuration of **38a** was confirmed by ^1H NMR, melting point and optical rotation.



i. BnOH, 80 °C.

Figure 3-8. Separation of isomeric benzyl esters



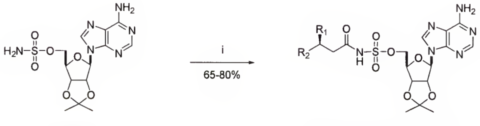
i. BnOH, 100 °C; ii. CbzCl, NaOH

Figure 3-9. Synthesis of aspartate analogs **39** and **40**

Aspartate analogs **39** and **40** were prepared using simple transformations (Figure 3-9). Opening of succinic anhydride **47** with benzyl alcohol yielded monobenzyl succinate **39** in excellent isolated yield (>95%).[78] Protection of the amino group in β-alanine was required to eliminate any interference in subsequent coupling reaction. This was accomplished by standard procedure using carbobenzyloxy chloride and sodium hydroxide.[79,80,81]

Synthesis of *N*-acyladenosinesulfamates.

With all components for a first series of inhibitors in hand, we decided to use a carbodiimide methodology to acylate the adenosinesulfamate with corresponding carboxylic acids. This was accomplished using *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI) as a coupling reagent.[82,83,84] After studying a number of different conditions and reagent combinations, it was apparent that the best results were obtained by using catalytic *N,N*-dimethylaminopyridine (DMAP) to shorten the reaction times and benzotriazole hydroxide (BtOH) in order to prevent accidental epimerization of formed acyl intermediate (in case of using **38a**). Formation of the acyl intermediate was accomplished by reacting the appropriate carboxylic acid **38a**, **39** or **40** with EDCI in the presence of DMAP and BtOH in anhydrous acetonitrile, at room temperature. The quality of the coupling reagent, EDCI was found to be crucial for this step. The acyl intermediate was intercepted *in situ* by adenosinesulfamate **44** to yield fully protected intermediates **49**, **50** and **51** (Figure 3-10).



44

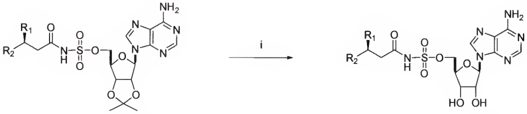
49 R_1 = NHCbz, R_2 = COOBn
50 R_1 = H, R_2 = COOBn
51 R_1 = NHCbz, R_2 = H

i. **38a**, **39** or **40**, EDCI, BtOH, DMAP, CH_3CN, 0°C to RT;

Figure 3-10. Synthesis of fully protected intermediates **49**, **50** and **51**

Isolated yields for this step were in range of 65-80%. As stated before, the purity of **44** was found to play an important role in the result of this reaction. It was necessary to purify small amounts of previously prepared **44** before repeating this step. Each of the isolated intermediates was purified and fully characterized before the next step.

Attempts to accomplish the next step (deprotection of acetonide moiety) were first carried out using Dowex 50-W resin as a proton source.[85] Use of this resin in water and methanol yielded only partial conversion to the desired diol even after long (72 hours) reaction times. Additionally, long exposure of **49**, **50** and **51** to acidic conditions caused decomposition of starting material. Furthermore, use of Amberlyst IR-120 resin did not improve the yield of this reaction. It became apparent that, in order to obtain the desired product and avoid decomposition, we would need to somehow reduce reaction times. Use of higher concentrations of acid seemed like a good choice for this transformation. Use of different concentration of HCl in THF[86] or water[87] and TFA in THF[88] yielded similar results; low rate of conversion and/or decomposition. Finally, by employing 50% TFA in water[88] we were able to obtain excellent yields using short reaction times (Figure 3-11).



49 R_1 = NHCbz, R_2 = COOBn
50 R_1 = H, R_2 = COOBn
51 R_1 = NHCbz, R_2 = H

52 R_1 = NHCbz, R_2 = COOBn
53 R_1 = H, R_2 = COOBn
54 R_1 = NHCbz, R_2 = H

i. 50% TFA/H_2O

Figure 3-11. Deprotection of acetonide; formation of **52**, **53** and **54**

With intermediates **52**, **53** and **54** in hand, we approached the final step – cleavage of benzyl and carbobenzyloxy protecting groups. Unfortunately, the first attempt using hydrogenolysis with 10% Pd/C as a catalyst[89] resulted in recovery of starting material; no progress in the reaction was observed whatsoever. Other catalysts, such as Pd black, $Pd(OH)_2$ and W2 Raney Nickel[90] did not drastically improve the output of this reaction. We suspected that even the non-nucleophilic sulfur in **52**, **53** and **54** may have been responsible for poisoning the catalyst, causing the lack of or slow reaction progress. Additionally, we have noticed that extended reaction times required by low activity of catalysts, and hydrogenolysis at increased pressure resulted in decomposition of starting material and any product formed. Hence, we attempted catalytic transfer hydrogenation – a method that is reported to cleave benzyl and carbobenzyloxy groups even in the presence of sulfur atom in the substrate.[91,92,93] Studies on various combinations of catalysts ($Pd(OH)_2$, Pd black, 10% Pd/C) and hydrogen donors (cyclohexene, 1,4-cyclohexadiene, formic acid, isopropanol) at various temperatures (room temperature or reflux) did not yield satisfactory results. Clean and complete reaction was only observed in small scale (2-5 mg) reactions using 10% Pd/C or 20% $Pd(OH)_2$ in ethanol/tetrahydrofuran mixture with 1,4-cyclohexadiene as hydrogen donor. The fact that a large excess of catalyst was required makes this method ineffective for large scale runs and further proved that poisoning the catalyst has occured. Moreover, most of the conditions investigated resulted in isolation of products that were contaminated with significant amounts of decomposition products that proved difficult to be remove by various chromatography methods. Additionally we observed that attempts to cleave benzyl and carbobenzyloxy groups in nucleophilic conditions using trimethylsilyl iodide[94,95] and HBr in acetic acid[96] resulted in complete decomposition of starting material. The same results were recorded for cleavage attempts in concentrated acids such as trifluoroacetic acid (TFA)[97] and trifluoromethanesulfonic acid (TFMSA).[98] Tables 3-1, 3-2 and 3-3 summarize the deprotection attempts.

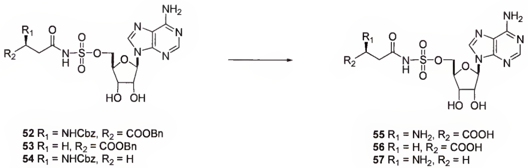

52 R_1 = NHCbz, R_2 = COOBn
53 R_1 = H, R_2 = COOBn
54 R_1 = NHCbz, R_2 = H
55 R_1 = NH_2, R_2 = COOH
56 R_1 = H, R_2 = COOH
57 R_1 = NH_2, R_2 = H

Figure 3-12 Deprotection attempts of **52**, **53** and **54**

Table 3-1. Deprotection attempts of **52**, **53** and **54** using hydrogenolysis

Catalyst	Solvent	Temp.	H donor	Conversion
10% Pd/C	EtOH	RT	H_2	No Reaction
10% Pd/C (excess)	MeOH	RT	H_2	100%
20% $Pd(OH)_2$	MeOH	RT	H_2	50%
20% $Pd(OH)_2$	MeOH	RT	H_2, 15 PSI	50%, Decomp.
10% Pd/C	MeOH	RT	H_2, 30 PSI	50%, Decomp.
10% Pd/C	EtOH /AcOH	RT	H_2	Decomp.
Pd black	MeOH	RT	H_2	50%
Pd black	MeOH	RT	H_2, 15 PSI	50%, Decomp.
Pd black	10% AcOH/ MeOH	RT	H_2	100%, Decomp.
Pd black	AcOH (glacial)	RT	H_2	100%, Decomp.

Table 3-1 continued

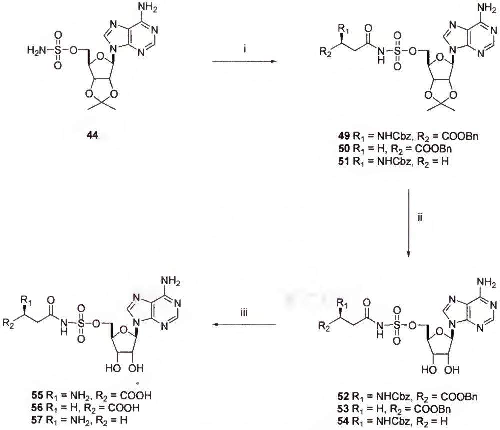
Pd black	1% HCl/ MeOH	RT	H_2	100%, Decomp.
Pd black	**10% TFA/ MeOH**	**RT**	**H_2**	**100%**

Table 3-2. Deprotection attempts of **52**, **53** and **54** using catalytic transfer hydrogenation

Catalyst	Solvent	Temp.	H donor	Conversion
20% $Pd(OH)_2$ (excess)	EtOH	RT	1,4-Cyclohexadiene	100%
20% Pd $(OH)_2$	MeOH	RT	Formic Acid	No Reaction
20% Pd $(OH)_2$	MeOH	Reflux	Formic Acid	Decomp.
10% Pd/C (excess)	EtOH /THF	Reflux	1,4-Cyclohexadiene	100%
10% Pd/C	MeOH	Reflux	Formic Acid	Decomp.
Pd black	Ethanol	RT	1,4-Cyclohexadiene	No Reaction
Pd black	MeOH	RT	Formic acid	50%
Pd black	MeOH	Reflux	Formic acid	Decomp.
Pd black	iPrOH	Reflux	Isopropanol	No Reaction

Table 3-3. Deprotection attempts of **52**, **53** and **54** using other methods

Reagent	**Solvent**	**Temp.**	**Result**
TMSI	CH2Cl2	RT	Decomp.
TMSI	MeCN	RT	Decomp.
TFA conc.	Neat	RT	Decomp.
HBr/AcOH	Neat	RT	Decomp.
TFMSA/TFA/m-cresol/DMS	Neat	RT	Decomp.
TFMSA	Neat	RT	Decomp.



44

49 R_1 = NHCbz, R_2 = COOBn
50 R_1 = H, R_2 = COOBn
51 R_1 = NHCbz, R_2 = H

52 R_1 = NHCbz, R_2 = COOBn
53 R_1 = H, R_2 = COOBn
54 R_1 = NHCbz, R_2 = H

55 R_1 = NH_2, R_2 = COOH
56 R_1 = H, R_2 = COOH
57 R_1 = NH_2, R_2 = H

i. **38a**, **39** or **40**, EDCI, BtOH, DMAP, CH_3CN, 0°C to RT;

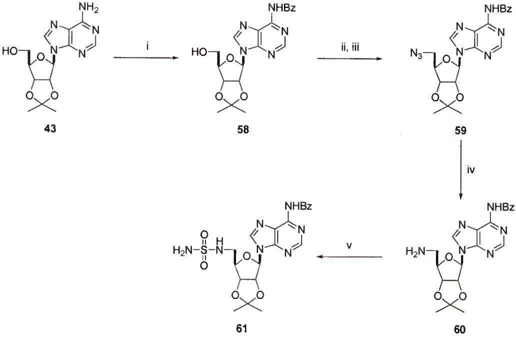
(ii) TFA/H_2O, rt; (c) Pd black, H_2, 5% TFA/MeOH, rt.

Figure 3-13. Summary of **55**, **56** and **57** synthesis

At one point in our analysis we had noticed that hydrogenolysis using Pd black as catalyst in mixtures of methanol and various acids results in fast consumption of starting material. When acetic acid (AcOH) and hydrochloric acid (HCl) were used, the starting material was completely consumed in 30 minutes, although decomposition was still observed. Switching the acid to trifluoroacetic acid (TFA) resulted in shorter reaction times. Additionally, the purity of isolated by filtration through celite products **55**, **56** and **57** was determined to be >95% (^{1}H NMR). The final purification of **55**, **56** and **57** was accomplished by chromatography using the Mitsubishi HP20SS resin.[50] The summary of the synthesis of **55**, **56** and **57**[99] is presented in Figure 3-13.

Synthesis of *N*-aspartateadenosinesulfamide.

In our approach to the N-acyladenosinesulfamide analog, protection of the 5-amino group in adenosine was necessary in order to distinguish two different amino groups (5- and 5'-) in further coupling reaction. This was accomplished by using a standard procedure in which 2,3-*O*-propylidene adenosine **43** was reacted with benzoyl chloride in the presence of pyridine and *N*,*N*-dimethylpyridine (DMAP) in dichloromethane (Figure 3-14).[100,101,102]



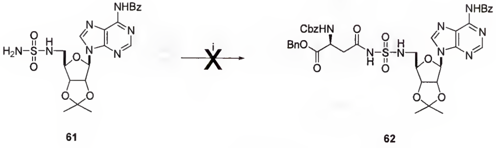
i. BzCl, Py, DMAP, DCM; ii. CBr_4, PPh_3, DMF; iii. NaN_3, DMF;

iv. H_2, Pd black, MeOH; v. Sulfamoyl chloride, Et_3N, CH_2Cl_2

Figure 3-14. Synthesis of intermediate 2,3-*O*-propylidene adenosinesulfamide

Benzoyl protected 2,3-*O*-propylidene adenosine **58,** isolated in 70% yield was further reacted with carbon tetrabromide in presence of triphenylphosphine. The bromide formed was reacted *in situ* with sodium azide to yield the corresponding azide **59** in 75% combined yield.[50] Hydrogenation of **59** in the presence of 10% Pd/C resulted in isolation of the target amine **60** suitable for 5'-*N*-sulfamide synthesis.[50] Conversion of **60** to the corresponding sulfamide **61** was accomplished by using sulfamoyl chloride and triethylamine (Et_3N) in dichloromethane.[50]

Initial run in which sulfamide **61** was reacted with *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI) and protected aspartate **38a** in the presence of *N*,*N*-dimethylaminopyridine (DMAP) and hydroxybenzotriazole (BtOH) did not afford the expected coupled intermediate **62** (Figure 3-15).

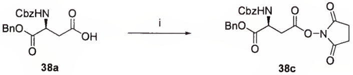


i. **38a**, EDCI, DMAP, BtOH, MeCN

Figure 3-15. Attempted coupling of **61** and **38a**

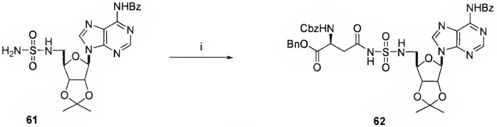
Presumably, the presence of the nitrogen atom in **61** (in place of oxygen atom in **44)** deactivates the sulfamide $-NH_2$ group used in coupling conditions. Hence, it was crucial to change the coupling protocol for this reaction. We decided to use a published methodology for coupling of deactivated amide $-NH_2$ group with *N*-succinamide esters in the presence of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU).[50,103] In order to use this method, it was necessary to convert protected aspartate **38a** to the corresponding *N*-succinimide ester **38c** (Figure 3-16).[104,105]

Published conditions for the formation of analogous esters were used for this reaction. Hence, **38a** was reacted with *N*-hydroxysuccinamide in presence of dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (BtOH).[106,107,108] *N*-succinimide ester **38c** was isolated in very good yield (79%) and fully characterized. Coupling of **61** and **31c** using DBU in DMSO resulted in isolation of the fully protected intermediate **62** in 72% yield (Figure 3-17).[50,103]



i. *N-hydroxy*succinamide, DCC, BtOH, DMSO.

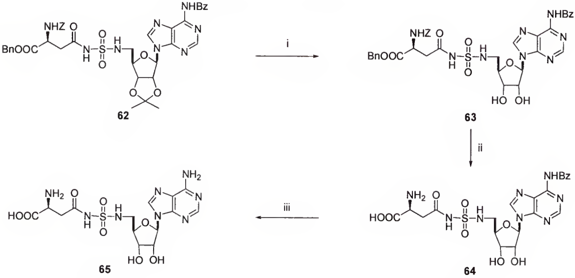
Figure 3-16. Synthesis of *N*-succinimide ester **38c**



i. **38c**, DBU, DMSO

Figure 3-17. Synthesis of fully protected intermediate **62**

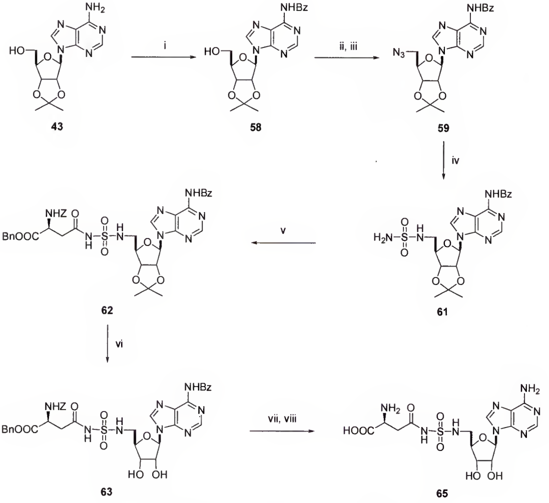
With **62** in hand, it was only necessary to decipher the right deprotection sequence. We decided that the cleavage of benzamide would be the last step of our synthesis; otherwise commonly used conditions (nucleophilic amines or base)[109,110,111] would most likely interfere with the benzyl ester protecting group in the aspartate moiety. Hence, we used an approach previously tested in the synthesis of sulfamates **55**, **56** and **57**. Treatment of **62** with 50% trifluoroaceticacid (TFA) in water[88] resulted in isolation of diol **63** in 95% yield. Subsequent hydrogenolysis using 10% TFA in methanol and palladium black as a catalyst afforded intermediate **64** with only the benzamide as the remaining protecting group (Figure 3-18).



i. TFA/H_2O; ii. H_2, Pd black, 10% TFA/MeOH; iii. *n*-$BuNH_2$/MeOH or NH_4OH/Dioxane or NH_4OH aq.

Figure 3-18. Synthesis of **64**

In our initial attempt, **64** was treated with n-butylamine in methanol according to a published procedure for removal of benzamide protecting group.[109] These reaction conditions yielded complete conversion to desired fully deprotected **65** in 7 hours (based on TLC). A simultaneous run using aqueous ammonium hydroxide[111] afforded comparable results.



i. BzCl, Py, DMAP, DCM; ii. CBr_4, PPh_3, DMF; iii. NaN_3, DMF

iv. H_2, Pd black, MeOH; v. $BnOOCCH(NHCbz)CH_2COOSucc$, DBU, DMSO;

vi. TFA/H_2O; vii. H_2, Pd black, MeOH/TFA; viii. NH_4OH

Figure 3-19. Complete synthesis of sulfamide **65**

^{1}H NMR analysis of both isolated samples revealed presence of significant amounts of impurities that proved difficult to remove. Fortunately, further reactions in which using concentrated aqueous ammonium hydroxide, afforded samples of **65** that proved to be

>95% pure based on ^{1}H NMR. Final purification was accomplished using the Mitsubishi HP20SS resin.[50] Figure 3-19 summarizes synthesis of sulfamide **65**.

Synthesis of *N*-acyladenosinesulfamates with Pyrrolidine-Based Aspartate Analog.

In the process of the design and synthesis of analogs of intermediates formed in enzymatic reactions, it was necessary to understand the role of particular structural moieties in binding to the active site. For this reason, we decided to further turn our attention to modifications of the aspartate moiety. As mentioned before, Parr[67] used pyrrolidine-based aspartate analogs **67a and 67b** to mimic the conformation of enzyme bound aspartate (Figure 3-20).

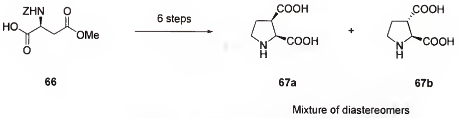
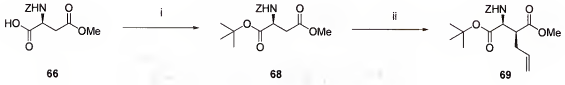


Figure 3-20. Aspartate analogs **67a** and **67b**

Free carboxylic diacids **67a** and **67b** can be prepared as a mixture of diastereoisomers in six steps from commercially available aspartate **66**. For the purpose of synthesizing βAspAMP analogs, it was necessary to modify the published procedure since the diacids **67a** and **67b** were not suitable for our coupling protocol. We wanted to accomplish a similar synthesis in which analogs with protected α- and free β- carboxyl could be accessed. We decided that the first four steps of Parr's[67] synthesis were suitable for our purpose. Only the last deprotection sequence needed to be modified. Hence, asparate **66** was reacted with isobutene according to literature conditions, to yield fully protected aspartate analog **68** in 95% yield (Figure 3-21).[112]

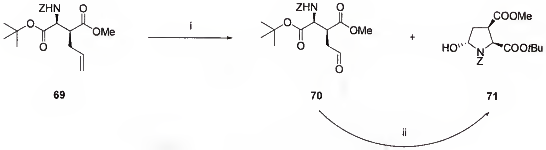


i. Isobutene, H_2SO_4, CH_2Cl_2; ii. *n*BuLi, HMDS, Allyl bromide, THF.

Figure 3-21. Synthesis of aspartate analog **69**

Treatment of **68** with *n*-butyllithium in presence of hexamethyldisilazene (HMDS) followed by allyl bromide yielded **69** as the only detectable diastereoisomer in 63% yield (Figure 3-20). No epimerization was observed at the chiral α-carbon, which was in agreement with previously published work. [113,114,115] Additionally, it was noted that workup conditions developed in our laboratory[67] proved to be crucial in order to obtain high diastereoselectivity in this reaction. The alkylation reaction was run at -78 °C and kept at this temperature overnight without warming up. After the alkylation of **68** was completed (as determined by TLC), the reaction had to be reversibly quenched by 1N HCl at room temperature. This particular sequence resulted in formation of **69** as the only diastereoisomer. Oxidative cleavage of the double-bond was carried out using periodate and a catalytic amount of osmium tetroxide. [116,117] This procedure yielded a mixture of pyrrolidine **71** and corresponding aldehyde **70**, presumable an intermediate in this transformation (Figure 3-22).

Reported yields for **70** and **71** were 6% and 83% respectively. In our case, the crude mixture contained a 1:1 ratio of **70** and **71**. Both components were easily separated by flash column chromatography. Additionally, we have established that overnight stirring of isolated aldehyde **70** in 50% methanol in water results in complete conversion into desired **71**.

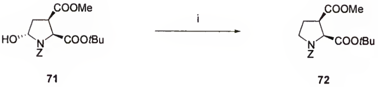


i. cat. OsO_4, $NaIO_4$, $MeOH/H_2O$; ii. $MeOH/H_2O$.

Figure 3-22. Oxidative double bond cleavage in **69**

It is also noteworthy that only a single stereoisomer of **71** was isolated, confirming the previous report fact that cyclization to form the desired pyrrolidine proceeds with complete control at C-5. The structure and configuration of **71** was confirmed by ^1H NMR with observed J_{23} coupling of 8.5 Hz, typical for *cis*-arranged pyrrolidine.

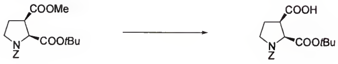
Reduction of **71** with triethylsilane in the presence of trifluoroacetic acid (TFA) in dichloromethane resulted in formation of **72** in 62% isolated yield (Figure 3-23).



i. Et_3SiH, TFA, CH_2Cl.

Figure 3-23. Reduction of **71**

According to the published synthesis,[67] **72** was next deprotected in two steps. First, the *tert*-butyl ester was cleaved using TFA, followed by cleavage of the methyl ester under basic conditions. Unfortunately, it was reported that the final steps resulted in epimerization of C-3 center and isolation of a 3:2 mixture of corresponding diastereoisomers, with the desired *cis*- diacid being the minor product. For the purpose of our synthesis direct deprotection of methyl ester in **72** was necessary. This would yield a α-protected acid **73** suitable for coupling with adenosinesulfamate. Additionally, we wanted to avoid epimerization of obtained product and associated problems with isolation of diastereoisomers. We decided to test different methods to accomplish deprotection of methyl ester without epimerization (Table 3-4).[118,119,120,121,122,123,124]



72 **73**

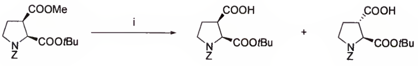
Figure 3-24 Deprotection attampts of **72**

Table 3-4. Attempts of deprotection of **72**

Method	**Result**
K_2CO_3 in THF/H_2O[118]	Deprotection of both esters
BCl_3 or boron catechol chloride in CH_2Cl_2, 0°C[119]	No reaction, slight decomposition
Trimethylsilyliodide (TMSI), CH_2Cl_2, 0°C[120]	Deprotection of methyl ester and carbobenzyloxy (Cbz) group
Pig Liver Esterase (PLE) pH 7 or 8[121,122,123]	No reaction
LiOH in $MeOH/H_2O$[124]	100% completion, racemization

Treatment of **72** with potassium carbonate in mixture of tetrahydrofurane and water[118] resulted in deprotection of both methyl and *tert*-butyl ester. When boron chloride or boron catechol chloride[119] was used, the starting material was completely recovered.

Use of trimethylsilyliodide (TMSI)[120] yielded a compound with both the methyl ester and carbobenzyloxy (Cbz) groups deprotected. Application of Pig Liver Esterase (PLE), an enzyme reported to cleave only one of symmetrical methyl ester groups[121,122,123] failed to display any reaction progress. We finally used lithium hydroxide in methanol and water.[124] This procedure resulted in isolation of mixture of **73a** and its diastereoisomer **73b** (Figure 3-25).



72 **73a** **73b**

i. LiOH, THF/H_2O.

Figure 3-25. Deprotection of **72**

Based on the ^{1}H NMR analysis, the ratio of the diastereomeric mixture was 5:3 with the desired diastereoisomer **73a** being the minor component. This conclusion was based on the analysis of ^{1}H NMR signal of H-2 and values of J_{23} coupling (Figure 3-26).

Two separate signals, from the proton attached to C-2 were observed in ^{1}H NMR, each one from diastereoisomers **73a** and **73b**. The J_{23} coupling value for the major component was observed to be 3.7 Hz which is consistent with the *trans-* arrangement of protons on C-2 and C-3[125, 126] and corresponds to structure **73b**. The minor component's J_{23} coupling was equal to 8.8 Hz, consistent value with structure **73a**, where the protons on C-2 and C-3 are in *cis-* configuration.

Attempts to separate **73a** and **73b** using standard chromatographic protocols failed; therefore, we decided to proceed with further steps using the mixture of diastereoisomers

73a and **73b**. EDCI coupling[82,83,84] of adenosinesulfamate **44** with mixture of **73a** and **73b** in the presence of DMAP and BtOH yielded fully protected diastereomeric intermediates **74a** and **74b** in 68% combined yield (Figure 3-27).

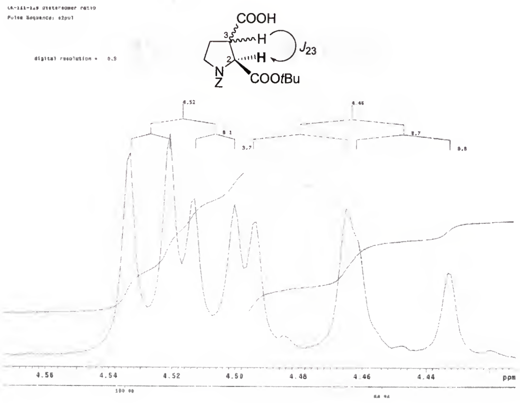
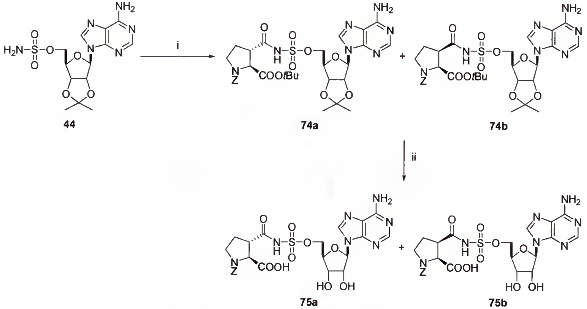


Figure 3-26. ^{1}H NMR determination of the diastereomeric mixture ratio

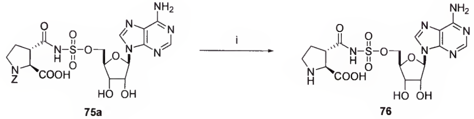
Treatment of the crude mixture of **73a** and **73b** with 50% TFA in water[88] resulted in cleavage of the acetonide. By extending the reaction time we were able to cleave the *tert*-butyl ester in the same step. Compounds **75a** and **75b** were isolated in 71% yield after two steps. Use of TFA also resulted in further epimerization of C-3 center to form the *trans* diastereoisomer **75a**. At the end, the crude mixture contained less than 10% of corresponding *cis*- product **75b**. Purification using reverse phase HPLC on C-18 column resulted in isolation of **75a** as a pure diastereoisomer. Final deprotection step was carried out using the previously established procedure. Hydrogenation using Pd black in 10% TFA in methanol resulted in isolation of fully deprotected **76** in 95% yield (Figure 3-28).



i. **73a** and **73b** mixture, EDCI, BtOH, DMAP, CH_3CN, 0°C to RT

ii. TFA/H_2O, Reverse Phase HPLC

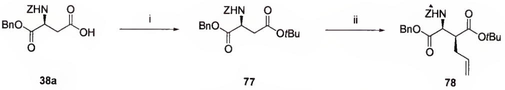
Figure 3-27. Synthesis of **74a** and **74b**



i. H_2, Pd black, 10% TFA in MeOH

Figure 3-28. Final step in synthesis of **76**

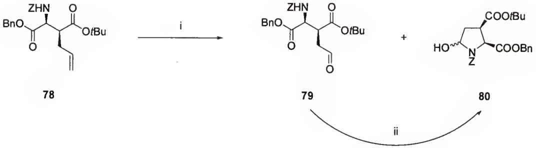
^{1}H NMR analysis of crude **76** revealed >95% purity of the sample. The final purification was accomplished using the Mitsubishi HP20SS gel.[50] Although the synthesis of **76** was successful, the configuration of the chiral centers in the amino acid analog did not correspond to those in aspartate. Unfortunately, almost complete epimerization at C-3 center in **75a** prevented us from isolating sufficient amount of its C-3 epimer **75b** suitable for further steps. Hence, it was necessary to develop a new synthesis of another βAspAMP analog with the correct *cis* configuration of C-2 and C-3 centers. By analyzing the synthesis of **76,** we came to the conclusion that the major weakness preventing us from obtaining the correct diastereoisomer was the methyl ester protecting group. Our inability to cleave this group under anything other than basic conditions, which resulted in epimerization, was the major problem. Therefore, we decided to use different protection pattern in our synthesis that would allow us to access the final product without using basic conditions. Previously synthesized[76,77] in large quantities and used by us protected aspartate **38a** seemed like a suitable starting material. Consequently, **38a** was converted to corresponding *tert*-butyl ester in 84% isolated yield, by reaction with isobutene in presence of acid catalyst (Figure 3-29).[127,128,129,130]



i. Isobutene, H_2SO_4, CH_2Cl_2; ii. HMDS, *n*BuLi, Allyl bromide, THF

Figure 3-29. Synthesis of **78**

Fully protected aspartate **77** was reacted with allyl bromide using the previously described method (Figure 3-29).[67] Compound **78** was the only formed and detected diastereoisomer. Its configuration was determined by data comparison to previously reported in the literature, and synthesized by us **69**.[67] Oxidative cleavage of the double bond in the presence of osmium tetroxide[116,117] resulted in the isolation of a mixture of desired hydroxypyrrolidine **79** and aldehyde **80** (Figure 3-30).

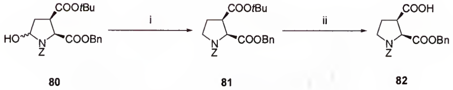


i. cat. OsO_4, $NaIO_4$, $MeOH/H_2O$; ii. $MeOH/H_2O$.

Figure 3-30. Oxidative cleavage of **78**

By overnight stirring of **79**, presumably an intermediate in the closure to the desired hydroxypyrrolidine in water/methanol mixture, we were able to achieve complete conversion to **80** (overall yield 54%). The control of the configuration at C-5 was not investigated, although based on ^1H NMR data we can suspect that **80** was synthesized as a mixture of distereoisomers at C-5. At this point this was irrelevant given that in the next step **80** was reduced to corresponding pyrrolidine **81** using triethylsilane and TFA (Figure 3-31).

Deprotection of the *tert*-butyl ester was achieved using TFA in dichloromethane.[131] Product **82** was isolated in 91% yield. Based on the ^1H NMR analysis of the J_{23} coupling value for the C-2 proton signal we concluded that **82** was the only detectable stereoisomer formed (Figure 3-32).



i. Et_3SiH, TFA, CH_2Cl_2; ii. TFA/ CH_2Cl_2

Figure 3-31. Synthesis of **82**

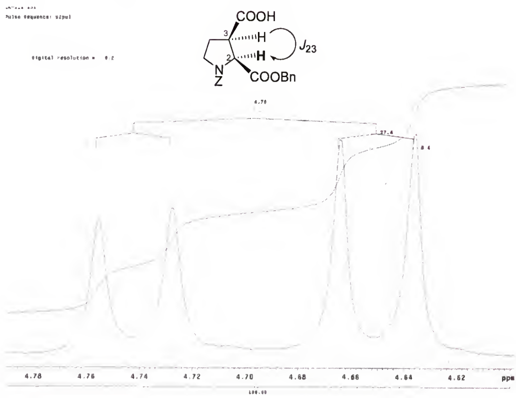
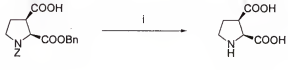


Figure 3-32. ^{1}H NMR analysis of **82** configuration

The observed J_{23} coupling value was 8.5 Hz, consistent for the reported values in *cis*-substituted pyrrolidine.[125,126] To confirm this observation, a small sample of **82** was fully deprotected to form previously reported in the literature **41** (Figure 3-33).[132,133,134]

Characterization data for **41** matched those published in the literature. Final structure conformation was accomplished by measuring the $[\alpha]_D$ of the formed diacid **41,** determined to be -41°. This was in absolute agreement with published data that reported it ranged from -41° to -42°.[132,133,134]



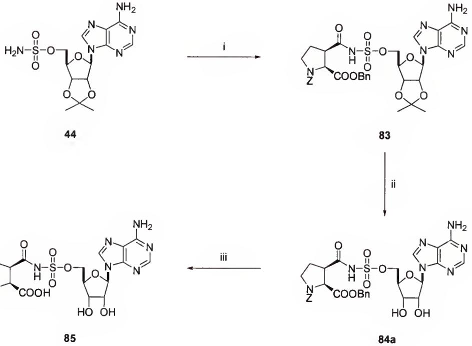
82

41

i. H_2, Pd black, TFA/MeOH

Figure 3-33. Full deprotection of **82**

With **82** in hand, we finished the synthesis of **85** using the same steps as those used in preparation of **76**. (Figure 3-34). Reaction of **44** and **82** was accomplished using EDCI coupling methodology.[82,83,84] Fully protected intermediate **83** was isolated in 66% yield. At this point, ^1H NMR analysis of the sample obtained did not reveal any epimerization of **83**. Therefore, we concluded that **83** was isolated as only one diastereoisomer. Cleavage of the acetonide moiety in 50% trifluoroacetic acid in water yielded diol **84a** in very good overall yield (90%). Unfortunately, the ^1H NMR spectrum revealed partial epimerization of C-3 center in the pyrrolidine (presumably caused by used strongly acidic conditions). It was necessary to separate both diastereomers formed using reverse-phase chromatography. The desired intermediate **84a** was isolated as a major component. The ratio of **84a** and its C-3 epimer **84b** was determined to be 9:1 based on HPLC. In the final step **84a** was hydrogenated using previously described conditions to afford the desired, fully deprotected **85**.

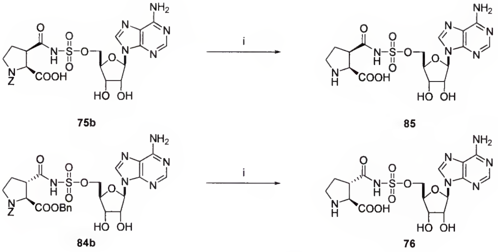


i. **82**, EDCI, BtOH, DMAP, CH_3CN, 0°C to RT;

ii. TFA/H_2O, Reverse Phase HPLC; iii. H_2, Pd black 10% MeOH/H_2O.

Figure 3-34. Synthesis of **85**

To confirm our speculations about the structure of the byproduct in the syntheses of **74a** and **84a**, we performed a complete deprotection of byproducts **74b** and **84b** isolated in the reverse-phase HPLC (Figure3-35). As expected, the collected data of the product isolated as a result of deprotection of **75b** matched the previously synthesized and characterized **85**. Similar results were observed for the reaction of **84b**, the final product was matched to **76**.

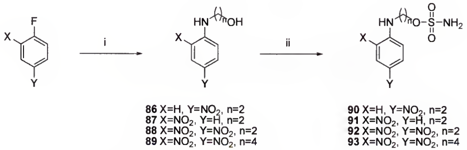


i. H^2, Pd black, 10% TFA/MeOH.

Figure 3-35. Deprotection of **75b** and **84b**

Synthesis of βAspAMP Analogs with Modified Adenosine Moiety.

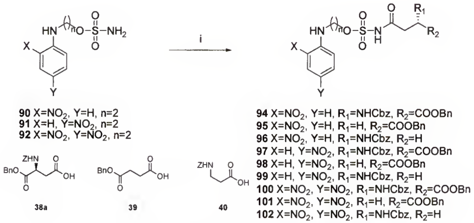
In the last phase of the synthesis of βAspAMP analogs, we attempted to synthesize compounds with non-nucleoside analogs of adenosine moiety. As mentioned before, we have chosen an array of different nitroaniline analogs for this purpose.[66] Four different analogs were prepared using Sanger's methodology (Figure 3-36).



i. 2-aminoethanol or 4-aminobutanol, THF; ii. NaH, sulfamoyl chroride, THF.

Figure 3-36. Synthesis of sulfamates **90**, **91**, **92** and **93**

Three different fluoro(di)nitrobenzenes were reacted with different aminoalcohols to yield alcohols **86-89** in excellent yields (>95%). Subsequent transformation to the corresponding sulfamates was carried out using previously tested procedure[71,72] and resulted in isolation of **90-93** (70-80% yield). The correct structure of the products was confirmed by comparison to published data. Additionally, the IR data collected ($1150cm^{-1}$ and $1350cm^{-1}$ stretch for H_2NSO_2O-) was consistent with the expected sulfamates **90-93**. It is noteworthy that the alternative method for preparation of sulfamates using *bis*-tributyltin oxide (BTO) and sulfamoyl chloride[73] did not provide satisfactory results. Similar problems to those described for the synthesis of **44** were observed. The EDCI coupling[82,83,84] of sulfamates **90, 91** and **92** with previously prepared aspartate analogs **38a, 39** and **40** yielded fully protected intermediates **94-102** (Figure 3-37).



90 $X=NO_2$, Y=H, n=2
91 X=H, $Y=NO_2$, n=2
92 $X=NO_2$, $Y=NO_2$, n=2

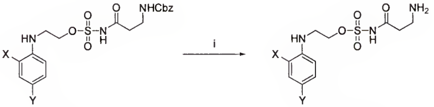
38a **39** **40**

94 $X=NO_2$, Y=H, R_1=NHCbz, R_2=COOBn
95 $X=NO_2$, Y=H, R_1=H, R_2=COOBn
96 $X=NO_2$, Y=H, R_1=NHCbz, R_2=H
97 X=H, $Y=NO_2$, R_1=NHCbz, R_2=COOBn
98 X=H, $Y=NO_2$, R_1=H, R_2=COOBn
99 X=H, $Y=NO_2$, R_1=NHCbz, R_2=H
100 $X=NO_2$, $Y=NO_2$, R_1=NHCbz, R_2=COOBn
101 $X=NO_2$, $Y=NO_2$, R_1=H, R_2=COOBn
102 $X=NO_2$, $Y=NO_2$, R_1=NHCbz, R_2=H

i. **38a** or **39** or **40**, EDCI, BtOH, DMAP, MeCN. 0°C to RT;

Figure 3-37. Synthesis of intermediates **94-102**

With fully protected intermediates **94-102** in hand, we proceeded to the final deprotection step. We have realized that the presence of an aromatic nitro group probably eliminated the possibility of cleaving the Cbz and benzyl ester using hydrogenolysis.[89] Attempted runs proved this a fact, and the nitro groups were readily reduced to corresponding amines. Hence, we have surveyed all existing methodology for cleavage of Cbz and benzyl ester groups in **94-102**. We have found that exposure of **94-102** to Lewis Acid conditions did not yield satisfactory results. Treatment with $AlCl_3$,[135] BBr_3,[136] BCl_3,[137] BF3*Et_2O[138] as well as catechol boron halides[139] in different solvent systems and temperatures resulted in rapid, complete decomposition of starting material. This fact can be explained by a number of different electrophilic centers that can be identified in the structures of **94-102**. Both carbon centers on the aminoethanol moiety are electron deficient. Additionally, formed as a result of nucleophilic attack on one of those centers nitroaniline and sulfamate anions are good leaving groups. Hence, it was unlikely that we would be able to achieve a selective attack on the benzylic positions in benzyl ester and carbobenzyloxy (Cbz) group. Fortunately, treatment of **96**, **99** and **102** with trimethylsilyl iodide (TMSI)[94,95,140] in acetonitrile yielded free amines **103**, **104** and **105** in 72-77% yield (Figure 3-38).



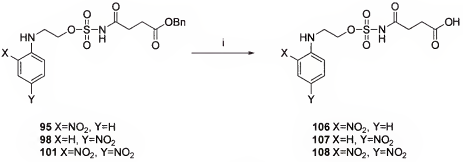
96 X=NO_2, Y=H
99 X=H, Y=NO_2
102 X=NO_2, Y=NO_2

103 X=NO_2, Y=H
104 X=H, Y=NO_2
105 X=NO_2, Y=NO_2

i. TMSI, MeCN, RT.

Figure 3-38. Final deprotection of **96**, **99** and **102**

Even though TMSI is also known to cleave benzyl esters, higher temperatures are required. Unfortunately attempts to deprotect **95**, **98** and **101** using this method at 50°C resulted in complete decomposition of the starting material. Therefore, benzyl esters **95**, **98** and **101** were hydrolyzed using 1.5 equivalent of potassium hydroxide in 50% THF in water[141] to yield the corresponding carboxylic acids **106**, **107** and **108** in 82-90% (Figure 3-39).

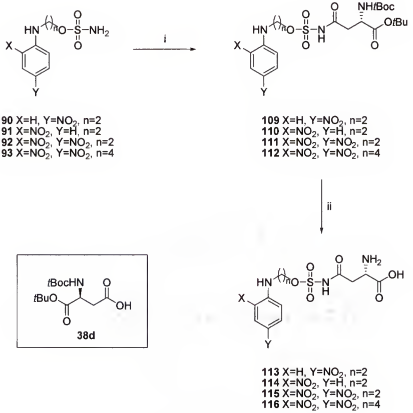


i. KOH, THF/H_2O.

Figure 3-39. Deprotection of **95**, **98** and **101**

Cleavage of the benzyl ester and carbobenzyloxy (Cbz) groups in **94**, **97** and **100** were accomplished by application of two subsequent, previously used deprotections – basic hydrolysis of the ester followed by TMSI cleavage of Cbz. Extremely low overall yields (<5%) and difficult purification processes encouraged us to find a new way for the preparation of the desired compounds. It was evident that the major drawbacks experienced in this synthesis were caused by problems with the removal of the protecting groups. Hence it was necessary to repeat the synthetic route using commercially available aspartate analog **38d**, in which the α-carboxyl and amino group are protected by acid labile groups (Figure 3-40). Previously described sulfamates **90-93** were coupled with

38d using EDCI methodology.[82,83,84]Fully protected intermediates **109-112** were isolated in 70-85% yields. Cleavage of *tert*-butyl ester and *tert*-butoxycarbonyl groups were completed using 0.05 M trifluoromethanesulfonic acid in 9/1 methylene chloride/dioxane mixture. The desired βAspAMP analogs **113-116** were isolated in 70-77% yields.



90 X=H, Y=NO_2, n=2
91 X=NO_2, Y=H, n=2
92 X=NO_2, Y=NO_2, n=2
93 X=NO_2, Y=NO_2, n=4

109 X=H, Y=NO_2, n=2
110 X=NO_2, Y=H, n=2
111 X=NO_2, Y=NO_2, n=2
112 X=NO_2, Y=NO_2, n=4

113 X=H, Y=NO_2, n=2
114 X=NO_2, Y=H, n=2
115 X=NO_2, Y=NO_2, n=2
116 X=NO_2, Y=NO_2, n=4

i. **38c**, EDCI, BtOH, DMAP, MeCN. 0°C to RT;

ii. 0.05M CF_3SO_3H in 9/1 CH_2Cl_2/Dioxane.

Figure 3-40. Synthesis of **113-116**

Conclusion

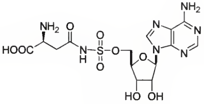
We were able to design and synthesize a library of 16 βAspAMP analogs with potentially interesting biological properties. In the process of preparation of this library we have encountered number of difficulties mainly associated with protecting groups in the aspartate moieties used in the synthesis. This was primarily caused by problems with obtaining aspartate analogs with proper protection pattern. Approaches to this type of aminoacids is often complicated, time consuming, and therefore, commercially available aspartates are high priced. Fortunately we were able to overcome those problems by developing suitable methods for deprotection as well as replacing benzyl (Bn) and carbobenzyloxy (Cbz) groups by acid labile *t*-butyl ester and *t*-butoxycarbonyl. All prepared compounds were fully characterized and purified before being used in the kinetic assays.

CHAPTER 4
BIOLOGICAL EVALUATION OF SYNTHESIZED ANALOGS

Specific Aims

Thanks to methodology recently developed in our laboratory, we have gained access to large quantities of human asparagine synthetase (hAS). We decided that by testing all synthesized analogs on human enzyme we will gain further insight on the structure of hAS active site and its kinetic mechanism, as well as on design of novel βAspAMP analogs specifically targeting the hAS. Hence, biological evaluation studies toward inhibition of hAS have been performed on each one of prepared compounds and obtained data was analyzed.

Prepared compounds are represented in figure 4-1. Each one of the 16 βAspAMP analogs displays certain characteristics that would allow us to probe the active site of hAS. Sulfamate **55** contains a structure similar to that of phosphoamidate **4**, with the exception of the sulfamate linker. Compounds **56** and **57** would enlighten the importance of hydrogen bonding of α-carboxyl and amino group to the hAS active site. Sulfamide **65** should explain the differences in active site interactions between the sulfamate or sulfamide linkers used in synthesis of βAspAMP analogs. Sulfamates **76** and **85** represent compounds that will be used in testing the ability of AS active site to accommodate additional groups attached to the aspartate moiety. Analogs **103-108** and **113-115** include novel non-nucleoside analogs of the adenosine moiety and therefore would answer questions about utility of those types of structures in novel drug-design process.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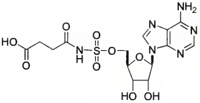
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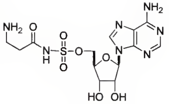
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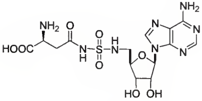
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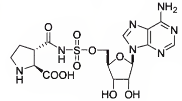
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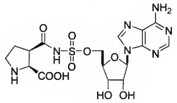
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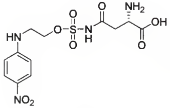
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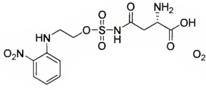
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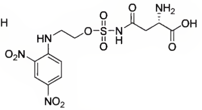
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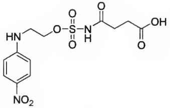
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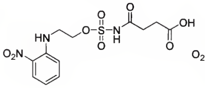
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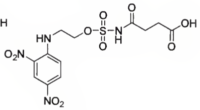
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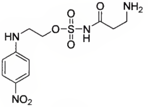
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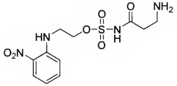
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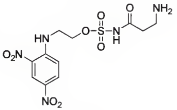
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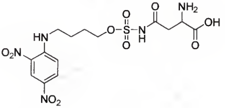

116

Figure 4-1. Synthesized βAspAMP analogs

Kinetic Assays

In attempts to analyze the kinetics of inhibitory action of the synthesized analogs, it is necessary to understand the mechanism of enzymatic catalysis of human asparagine synthetase (hAS). Because of the lack of detailed data on the human enzyme we have assumed that hAS displays similar kinetics to those observed for bacterial asparagine synthetase B (AS-B).

There are three distinct substrates for the synthetase active site: ammonia, aspartate, and ATP. As described in chapter one, in the first step aspartate is activated by ATP to form the βAspAMP intermediate. In this reaction, a molecule of pyrophosphate is released from the active site. In the following step, the intermediate is cleaved by ammonia and asparagine is released. For the purposes of detailed kinetic analysis of inhibitory action it is crucial to accurately measure the increase of the concentrations of products formed or decrease of concentrations of the substrates. Although procedures for accurate measurements of asparagine formed exist,[142] they are time consuming and inconvenient for screening of large number of compounds. Hence, we decided to monitor the concentration of pyrophosphate formed using standard procedures.[143] A commercially available assay mix[144] for measuring concentrations of pyrophosphate was used. The assay contains a system of four coupled enzymes in which two moles of NADH are oxidized to NAD^+ per one mole of pyrophosphate consumed. Decrease of NADH concentration over time can be easily monitored spectrophotometrically. This assay allows measurement of low concentrations of pyrophosphate formed (down to 10 nM). Additionally, measurements for a large number of continuous assays are possible. Moreover, recent findings[23,142] confirmed the stoichiometry of AS-B catalyzed reaction to be one to one with respect to asparagine and pyrophosphate formed.

Available data[21,22,23,142] suggests that the first substrate to bind to the synthetase active site is ATP. Considering the structures of our synthesized analogs, it is very likely that they bind to the same form of the enzyme as does ATP. Consequently, it is possible to observe decreased inhibitory activity in high concentrations of ATP due to competition for the same binding site. Previously reported conditions[145] (0.5 mM ATP, approx. 2.5 X K_m) were used in order to avoid this problem. Concentrations of the two remaining substrates (ammonia and aspartate) were kept at much higher levels than respective K_m values.

Results and Discussion

Inhibition Analysis of Sulfamate 55.

Sulfamate **55** displayed significant inhibition properties in the performed assays.

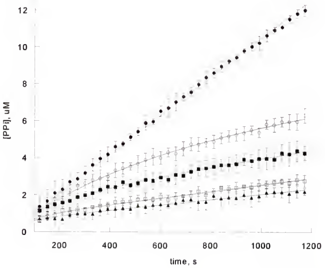


Figure 4-2. Progress curves for 0, 5, 10, 25 and 50 μM **55** at 0.5 mM ATP

This is presumably caused by binding within the C-terminal, synthetase site of hAS in a similar fashion to the βAspAMP intermediate formed in the catalytic process of asparagine production (Figure 4-2, data points represent experimental values, lines were fitted using equations for slow-onset inhibition).

Importantly, control experiments established that the pyrophosphate reagent is not affected by the presence of sulfamate **55**. Analysis of the steady state kinetics of hAS inhibition by **55** was undertaken to determine the mechanism by which the inhibitor exerted its effects. It was striking to observe that after initial linear dependence of formed pyrophosphate, in time, the inhibitory activity of **55** seemed to increase. The graphs for different concentrations of **55** resembled those typical for slow-onset inhibition.[146] Hence, the experimental data were best fit using following the equation corresponding to slow-onset inhibition:[146]

$$[PP_i] = v_{ss}t + [(v_o - v_{ss}) / k]\,[1 - \exp(-kt)]$$

where v_o is the initial velocity of the reaction, v_{ss} is the velocity at large t, and k is a parameter that depends on the inhibitor concentration. Further experiments demonstrated that elevated levels of ATP decreased the ability of **55** to inhibit recombinant hAS (Figure 4-3).

This implies that ATP and **55** compete for the free enzyme. This observation is consistent with the assumption that **55** binds within the C-terminal, synthetase site of AS. The inhibition mechanism is thus consistent with the following model:

$$\begin{tikzcd}
\mathrm{E} \arrow[r, shift left, "k_1\mathrm{A}"] \arrow[d, shift left] & \mathrm{EA} \arrow[l, shift left] \arrow[r, "k_7"] & \mathrm{E} \\
\mathrm{EI} \arrow[u, shift left, "\mathrm{K_i}"] \arrow[r, shift left, "k_5"] & \mathrm{EI^*} \arrow[l, shift left, "k_6"] &
\end{tikzcd}$$

for which the variation of k with inhibitor concentration is given by:

$$k = k_6 + k_5\,(I/K_i) / [1 + [ATP]/K_a + I/K_i]$$

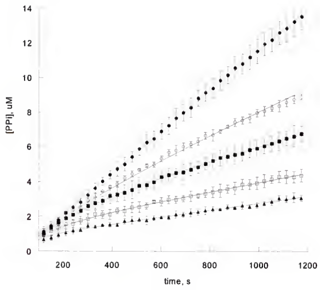


Figure 4-3. Progress curves for 0, 5, 10, 25 and 50 μM **55** at 5 mM ATP

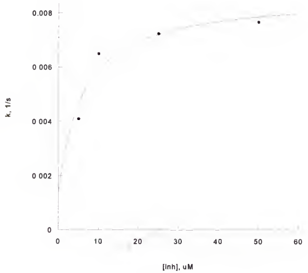


Figure 4-4. Plot of *k* versus [**55**] at 0.5 mM ATP

Analysis of the progress curves using the slow-onset inhibition equation gave the values of k as a function of the concentration of **55**. Replot of the k versus [**55**] (Figure 4-4) yielded values for K_i, k_5, and k_6 of 1.7 μM, $7.28 \times 10^{-3}\ s^{-1}$, and $1.4 \times 10^{-3}\ s^{-1}$ respectively. The overall dissociation constant (K_i*) for the slow-onset inhibitor **55** is therefore 280 nM.[146]

Under the similar experimental condition sulfamates **56** and **57** were only capable of inhibiting recombinant hAS at 100-fold greater concentrations (see APPENDIX A for inhibition data). Unfortunately none of the remaining synthesized βAspAMP analogs displayed any inhibitory activity toward hAS in concentrations as high as 500 μM. Although it may seem expected for analogs containing modified adenine moiety (analogs **103-108** and **113-116**), those results were somewhat surprising especially for analogs **65**, **76** and **85**.

Inhibition Analysis of Sulfoximine 5

With inhibition data for sulfamate **55** in hand we decided to perform an analogous study for the sulfoximine **5**[38] synthesized by Hiratake. Our results allowed us to compare two different types of inhibitors – intermediate and transition state analogs. Sulfoximine **5** displayed strong inhibition of hAS in the 0-10 μm concentration range (Figure 4-5). Control experiments eliminated any influence of **5** on pyrophosphate reagent. Similarly to sulfamate **55**, sulfoximine **5** exhibits increase of inhibitory activity as the time progresses, and decreased activity in higher ATP concentration (Figure 4-6). Hence, experimental data was fit using slow-onset inhibition equation. The plot of k in different concentrations of **5** is shown in Figure 4-7.

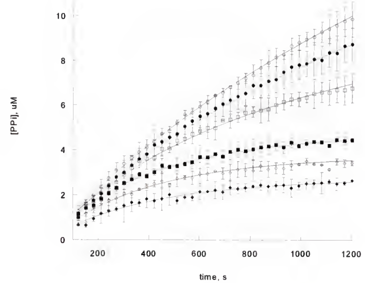


Figure 4-5. Progress curves for 0.5, 1, 2, 4, 6, and 10 μM **5** at 0.5 mM ATP

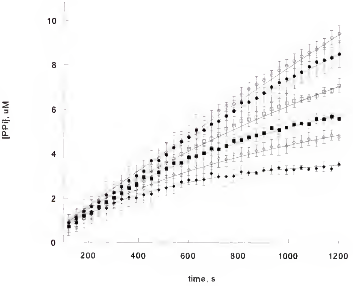


Figure 4-6. Progress curves for 0.5, 1, 2, 4, 6, and 10 μM **5** at 5 mM ATP

Observed values for K_i, k_5, and k_6 of 405 nM, $4.36 \times 10^{-3}\ s^{-1}$, and $1.65 \times 10^{-4}\ s^{-1}$ respectively. The overall dissociation constant (K_i^*) for the slow-onset inhibitor **5** is therefore 15 nM.[146]

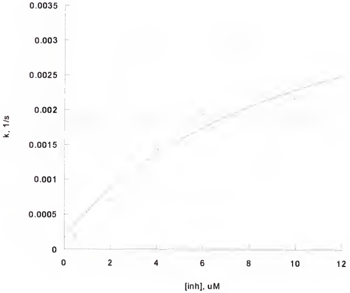


Figure 4-7. Plot of *k* versus [**5**] at 0.5 mM ATP

CHAPTER 5
CONCLUSION AND FUTURE WORK

We have synthesized and characterized the first inhibitor of human asparagine synthetase (hAS).[99] Sulfamate **55** exhibits slow-onset type inhibition of hAS with dissociation constant of 280 nM.[146] While still not sub-nanomolar in potency, sulfamate **55** represents a useful lead compound for future studies in this area. βAspAMP analogs **56** and **57,** where succinate and β-alanine moiety displaced the aspartate, displayed a 100-fold decrease in inhibition properties. This fact displays the importance of the aspartate amino- and carboxyl- groups in binding to hAS active site. Both groups most likely account for two hydrogen bonds formed in the process of docking to the active site of hAS, hence the 100-fold drop in inhibitory activity is explicable.

Lack of activity of sulfamide **65** was somewhat surprising, in view of the fact that it differs only by a nitrogen atom present in the linker instead of oxygen atom, when compared to **55**. Apparent explanations for these observations include possible changes in the favorable conformation as well as diminished hydrogen bonding – where the acceptor oxygen was replaced by donor nitrogen.

Another useful, but at the same time disappointing observation, was the lack of inhibitory activity of βAspAMP analogs **76** and **85**. This could be explained by the inability of the hAS active site to accommodate aspartate analogs bearing substituents at C-3. Similarly, βAspAMP analogs **103-108** and **113-116** with nitroaniline-*N*-alcohols as adenosine mimics did not display any inhibitory activity in concentrations up to 500 μM.

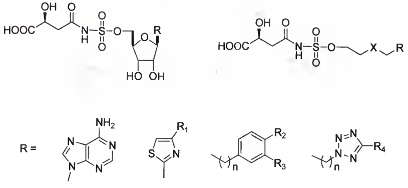
Hence, we believe that in the design of future βAspAMP analogs it is necessary to use adenosine, or mimics with hydrogen bond pattern not drastically modified from it.

As a comparison to inhibition properties of sulfamate **55** we screened previously reported sulfoximine **5** for inhibition activity toward hAS. **5** exhibits similar to **55** slow-onset type of inhibition kinetics with dissociation constant of 15 nM. Hence, it is approximately 20 times more potent than our sulfamate **55**. These data are in agreement with the general principle that transition state analogs (**5**) bind tighter to the enzyme active site than intermediate analogs (like **55**).

Based on our results, we can create guidelines for the design and synthesis of future βAspAMP analogs. We have established that the sulfamate linker used in **55** offers a clear advantage over phosphoramidate in **4**. Suitable for synthesis of different analogs sulfamate intermediates are available by simple chemical transformations. Therefore, complicated, low yield coupling conditions used in synthesis of **4** can be eliminated. Moreover, use of sulfamate linker results in formation of much more stable analogs.

Based on obtained results we came to conclusion that the aspartate mimics used in future βAspAMP analogs should bear the same hydrogen bonding pattern as aspartate, and lack any significant modifications of the carbon chain. Consequently, one can envision aspartate mimics with hydroxyl replacing amino group in future targets.

Based on the negative results from inhibition studies of analogs with a drastically modified adenosine group, it is necessary to investigate the importance of both ribose and adenine in binding to the active site of hAS. We therefore propose two different adenosine modifications to be used in future βAspAMP analogs. The first group would include the complete ribose moiety with a simplified heterocycle. In the second one adenine would be attached to a simplified ribose ring. Some of the proposed analogs, designed based on above facts are displayed in Figure 5-1.



X = O, CH_2; R = adenine, thiazole, tetrazole derivatives

R_1 = 4'-cyano-furanyl, 2'-OMe-Ph, 3'-OMe-Ph, PhOPh, 4'-OMe-Ph, 6'-OMe-2-naphtyl, CH_2CH_2Ph.

N = 1,2; R_2 , R_3 = OH, OMe; R_4 = amino, alkyl, aryl, cycloalkyl, aloxy, aryloxy

Figure 5-1. Structures of some novel βAspAMP analogs.

Further investigation of the inhibition kinetics to complete detailed analysis of **5** and **55** is also required. Especially, reactivation experiments of inhibited enzyme would provide an useful information and are necessary to confirm the proposed mechanism. Furthermore, βAspAMP analogs with confirmed inhibition properties toward hAS will be used as probes on actual acute lymphoblastic leukemia cell lines and in efforts to crystallize hAS.

CHAPTER 6
EXPERIMENTAL SECTION

Kinetic Assays

Biochemical Assays of AS Inhibition Progress curves were generated by incubating purified human AS (4) in reaction mixtures containing 100 mM HEPPS, pH 8.0, 100 mM ammonium chloride, 0.5 or 5 mM ATP, 10 mM aspartate, and 10 mM $MgCl_2$ in the presence of the appropriate concentration of different βAspAMP analogs. The velocity of each reaction was monitored spectrophotometrically by following the production of pyrophosphate during asparagine synthesis using pyrophosphate reagent at 37°C (Sigma Chemical Company, Technical Bulletin No. BI-100).

Compound Characterization

Melting points were recorded using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured using a Polyscience Model SR-6 polarimeter. ^1H and ^{13}C NMR spectra were obtained at 300 and 75.4 MHz, respectively. For ^1H and ^{13}C chemical shifts are reported in ppm (δ) downfield of tetramethylsilane as an internal reference (δ 0.0), except for measurements in D_2O for which acetone (δ 2.1) was employed as an external standard. Splitting patterns are abbreviated as follows: s, singlet, d, doublet, t, triplet, q, quartet and m, multiplet. EI, CI and FAB mass spectra were recorded on a Finnegan MAT 25Q (high resolution) spectrometer. Methane was generally employed in obtaining CI mass spectra. Analytical thin layer chromatography (TLC) was performed on silica gel 60F-254 plates. Flash chromatography was performed using standard procedures[147] on Kieselgel (230-400 mesh), and size exclusion chromatography was performed on Sephadex G-10 resin. Semi-preparative reverse-phase (C18) HPLC was carried out on a RAININ system using a DYNAMAX-60A column (2 cm x 30 cm). All reagents were purchased from Aldrich or Fisher Scientific, and were used without further purification except for chromatography solvents, which were distilled before use. Moisture sensitive reactions were carried out under a nitrogen, or argon, atmosphere in glassware that was flame-dried with an inert gas sweep. THF was freshly distilled from sodium benzophenone ketyl before use.

***N*-(carbobenzyloxy)-α-benzyl-aspartic acid *N*-succinimide ester (38c)**. A flame dried, nitrogen flushed 150 mL RBF was charged with 1g (2.8 mMol) of N-Cbz-α-benzyl aspartic acid **38a** in 50 mL of CH_2Cl_2. Catalytic amount of DMAP and hydroxybenzotriazole was added followed by N-hydroxysuccinimide (355 mg, 3.08 mMol) and DCC (635 mg, 3.08 mMol). The reaction mixture was stirred at room temperature for 1 h and cooled to 0°C. The solid residue was removed by filtration and solvent was removed under reduced pressure. Crude material was purified by flash column chromatography (silica gel 100g, CH_2Cl_2/MeOH 97/3) to yield 1g (79%) of **39c** as colorless oil. $[\alpha)_D^{23} = -13.7^o$ (c=0.81 in MeOH). IR (neat): ν 3345, 1813, 1783, 1737, 1515, 1203, 1060, 740, 697 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 7.35 (m, 10H), 5.86 (d, 1H, J = 8.1 Hz), 5.18 (m, 4H), 4.79 (m, 1H), 3.34 (dd, 1H, J = 17.1, 4.9 Hz), 3.22 (dd, 1H, J = 16.7, 4.8 Hz), 2.82 (s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.32 (s), 168.75 (s), 166.00 (s), 155.70 (s), 135.92 (s), 134.77 (s), 128.40 (d), 128.32 (d), 128.29 (d), 128.04 (d), 127.96 (d), 127.84 (d), 67.73 (t), 66.97 (t), 50.09 (d), 33.74 (t), 25.28 (t). MS (ES-FTICR): 493 ($M+K^+$, 15), 477 ($M+Na^+$, 100), 238 ($M+2H^+$, 4). HRMS (ES-FTICR): calcd for $C_{23}H_{22}N_2O_8Na$ 477.4261; found 477.1157.

4 - [6 - (6 – Amino – purin – 9 - yl) - 2,2 – dimethyl – tetrahydro – furo [3,4 - d] [1,3] dioxol – 4 - ylmethoxysulfonylamino] – 2 – benzyloxycarbonylamino – 4 – oxo - butyric acid benzyl ester (49). A flame dried, nitrogen flushed 25 mL RBF was charged with 280mg (0.78 mmol) of N-Cbz-α-benzyl aspartic acid **38a** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 150mg (0.78 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. Adenosine sulfamate **44** (200 mg, 0.52 mmol) in 5 mL of acetonitrile was added at 0°C. Reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 310mg (79%). White foam. $[\alpha)_D^{25}$ = -32.3° (c = 0.28 in MeOH). IR (neat): ν 3355, 2988, 1714, 1645, 1600, 1376, 1297, 1213, 1151, 1080, 853, 698 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.41 (s, 1H), 8.19 (s, 1H), 7.26 (m, 10H), 6.20 (d, 1H, *J* = 2.9 Hz), 5.24 (dd, 1H, *J* = 5.8, 2.9 Hz), 5.00 – 5.12 (m, 5H), 4.68 (m, 1H), 4.49 (m, 1H), 4.18 (m, 2H), 2.74 (m, 2H), 1.58 (s, 3H), 1.34 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 178.98 (s), 173.59 (s), 158.54 (s), 157.36 (s), 154.04 (d), 150.47 (s), 141.54 (d), 138.06 s), 137.24 (s), 129.59 (d), 129.51 (d), 129.20 (d), 129.20 (d), 129.05(d), 128.82 (d), 120.24 (s), 115.36 (s), 91.90 (d), 85.95 (d), 85.67 (d), 83.29 (d), 69.79 (t), 68.17 (t), 67.82 (t), 52.96 (d), 41.74 (t), 27.63(q), 25.72 (q). MS (FAB): 770 (M - H^+ + $2Na^+$, 90), 748 (M + Na^+, 20), 697 (7), 290 (100). HRMS (FAB): calcd for $C_{32}H_{35}O_{11}N_7SNa$ 748.2012; found 748.2027.

4 - [6 - (6 – Amino – purin – 9 - yl) - 2,2 – dimethyl – tetrahydro - furo[3,4 - d] [1,3] dioxol – 4 – ylmethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (50).

A flame dried, nitrogen flushed 25 mL RBF was charged with 160mg (0.78 mmol) of monobenzyl succinate **39** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 150mg (0.78 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. Adenosine sulfamate **44** (200 mg, 0.52 mmol) in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). Analytical sample was prepared using preparative scale TLC. Y = 200mg (66%). White foam. $[\alpha)_D^{25}$ = -26.7° (c = 0.29 in MeOH). IR (neat): ν 3345, 3210, 1728, 1651, 1600, 1455, 1383, 1213, 1149, 1045, 834 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.42 (s, 1H), 8.20 (s, 1H), 7.30 (m, 5H), 6.23 (d, 1H, *J* = 3.2 Hz), 5.32 (dd, 1H, *J* = 6.1, 3.2 Hz), 5.09 (dd, 1H, *J* = 6.1, 2.1 Hz), 5.06 (s, 2H), 4.52 (d, 1H, *J* = 2.1 Hz), 4.20 (d, 2H, *J* = 3.8 Hz), 2.61 (t, 2H, *J* = 6.2 Hz), 2.52 (t, 2H, *J* = 6.2 Hz), 1.60 (s, 3H), 1.38 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 181.13 (s), 174.88 (s), 157.43 (s), 154.06 (d), 150.61 (s), 141.51 (d), 137.78 (s), 129.58 (d), 129.11 (d), 129.11 (d), 120.20 (s), 115.30 (s), 92.01 (d), 85.90 (d), 85.76 (d), 83.49 (d), 69.70 (t), 67.34 (t), 34.72(t), 31.15 (t), 27.66 (q), 25.72 (q). MS (FAB): 621 (M - H^+ + $2Na^+$, 17), 599 (M + Na^+, 30), 577 (M + H^+, 40), 372 (7), 308 (15), 290 (50), 280 (100), 207 (25), 115 (40). HRMS (FAB): calcd for $C_{24}H_{29}O_9N_6S$ 577.1717; found 577.1708.

{3 - [6 - (6 – Amino – purin – 9 - yl) - 2,2 – dimethyl – tetrahydro – furo [3,4 - d] [1,3] dioxol – 4 - ylmethoxysulfonylamino] – 3 – oxo - propyl} - carbamic acid benzyl ester (51). A flame dried, nitrogen flushed 25 mL RBF was charged with 175mg (0.78 mmol) of N-Cbz-β-alanine **40** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 150mg (0.78 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. Adenosine sulfamate **44** (200 mg, 0.52 mmol) in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 195mg (63%). White foam. $[\alpha]_D^{25} = -17.6^{\circ}$ (c = 0.27 in MeOH). IR (neat): ν 3342, 2988, 1694, 1651, 1599, 1454, 1377, 1253, 1214, 1147, 1072, 834 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.41 (s, 1H), 8.19 (s, 1H), 7.28 (m, 5H), 6.22 (d, 1H, $J = 2.9$ Hz), 5.29 (dd, 1H, $J = 6.0, 3.1$ Hz), 5.10 (dd, 1H, $J = 5.8, 2.0$ Hz), 5.01 (s, 2H), 4.52 (d, 1H, $J = 2.1$ Hz), 4.22 (d, 2H, $J = 3.5$ Hz), 3.36 (t, 2H, $J = 6.4$ Hz), 2.38 (t, 2H, $J = 6.4$ Hz), 1.60 (s, 3H), 1.36 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 181.00 (s), 158.75 (s), 157.42 (s), 154.06 (d), 150.51 (s), 141.55 (d), 138.42 (s), 129.52(d), 129.00 (d), 128.86 (d), 120.26 (s), 115.37 (s), 91.93 (d), 85.99 (d), 85.85 (d), 83.31 (d), 69.69 (t), 67.47 (t), 40.15 (t), 38.85 (t), 27.63 (q), 25.68 (q). MS (FAB): 636 ($M - H^+ + 2Na^+$, 30), 614 ($M + Na^+$, 40), 603 (10), 369 (7), 290 (35), 155 (35). HRMS (FAB): calcd for $C_{24}H_{29}O_9N_7SNa$ 614.1645; found 614.1621.

4 - [5 - (6 – Amino – purin – 9 - yl) - 3,4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylamino] – 2 – benzyloxycarbonylamino – 4 – oxo - butyric acid benzyl ester (52). 42mg (0.058 mmol) of acetonide **49** was suspended in 2 mL of water. 2 mL of TFA were added. The reaction mixture was stirred at room temperature for 3 hours (monitored by TLC). Removal of solvent under reduced pressure afforded crude diol **52**. An analytical sample was purified using preparative scale TLC. Y = 33 mg (83%). White foam. $[\alpha]_D^{25} = -3.9^{\circ}$ (c = 0.25 in MeOH). IR (neat): ν 3456, 1686, 1648, 1207, 1140, 842 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.50 (s, 1H), 8.16 (s, 1H), 7.26

(m, 10H), 6.08 (d, 1H, *J* = 4.7 Hz), 5.00 - 5.14 (m, 4H), 4.68 (m, 1H), 4.60 (m, 2H), 4.36 (m, 1H), 4.28 (m, 2H), 2.78 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 179.01 (s), 173.70 (s), 158.61 (s), 157.38 (s), 153.94 (d), 150.75 (s), 141.30 (d), 138.07 (s), 137.23 (s), 129.59 (d), 129.52 (d), 129.52 (d), 129.23 (d), 129.07 (d), 128.78 (d), 120.37 (s), 89.45 (d), 84.42 (d), 76.36 (d), 72.17 (d), 69.20 (t), 68.24 (t), 67.82 (t), 52.94 (d), 41.81 (t). MS (FAB): 686 (M + H$^+$,10). HRMS (FAB): calcd for $C_{29}H_{32}O_{11}N_7S$ 686.1880; found 686.1911.

4 - [5 - (6 – Amino – purin – 9 - yl) - 3,4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (53). Acetonide **50** (40mg, 0.069 mmol) was suspended in 2 mL of water. 2 mL of TFA were added. The reaction mixture was stirred at room temperature for 3 hours (monitored by TLC). Removal of solvent under reduced pressure afforded crude diol **53**. An analytical sample was purified using preparative scale TLC. Y = 33 mg (89%). White foam. $[\alpha)_D^{25} = -14.0^o$ (c = 0.27 in MeOH). IR (neat): ν 3442, 1682, 1650, 1604, 1480, 1300, 1207, 1146, 845 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.48 (s, 1H), 8.18 (s, 1H), 7.28 (m, 5H), 6.08 (d, 1H, *J* = Hz), 5.05 (s, 2H), 4.64 (t, 1H, *J* = 5.5 Hz), 4.36 (m, 1H), 4.26 (m, 3H), 2.62 (t, 2H, *J* = 5.9 Hz), 2.54 (t, 2H, *J* = 5.9 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.24 (s), 175.10 (s), 157.45 (s), 154.02 (d), 150.99 (s), 141.31 (d), 137.78 (s), 129.59 (d), 129.46 (d), 129.14 (d), 120.39 (s), 89.47 (d), 84.64 (d), 76.27 (d), 72.41 (d), 69.23 (t), 67.41 (t), 34.84 (t), 31.27 (t). MS (FAB): 537 (M + H$^+$, 10), 510 (3), 418 (10), 364 (100), 290 (20), 250 (30). HRMS (FAB): calcd for $C_{21}H_{25}O_9N_6S$ 537.1404; found 537.1408.

{3 - [5 - (6 – Amino – purin – 9 - yl) - 3,4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylamino] – 3 – oxo - propyl} - carbamic acid benzyl ester (54).

Acetonide **51** (40 mg, 0.068 mmol) was suspended in 2 mL of water. TFA (2 mL) was added. The reaction mixture was stirred at room temperature for 3 hours (monitored by TLC). Removal of solvent under reduced pressure afforded crude diol **54**. An analytical sample was purified using preparative scale TLC. Y = 30 mg (80%). White foam. $[\alpha)_D^{25}$ = -10.4° (c = 0.25 in MeOH). IR (neat): ν 3358, 1681, 1648, 1604, 1294, 1206, 1139, 842 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.50 (s, 1H), 8.18 (s, 1H), 7.28 (m, 5H), 6.08 (d, 1H, *J* = 5.3 Hz), 5.00 (s, 2H), 4.61 (t, 1H, *J* = 5.3 Hz), 4.23 – 4.41 (m, 4H), 3.38 (t, 2H, *J* = 6.4 Hz), 2.40 (t, 2H, *J* = 6.4 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.08 (s), 158.86 (s), 157.46 (s), 154.00 (d), 150.92 (s), 141.36 (d), 138.48 (s), 129.55 (d), 129.02 (d), 128.84 (d), 120.43 (s), 89.62 (d), 84.53 (d), 76.33 (d), 72.24 (d), 69.09 (t), 67.49 (t), 40.24 (t), 38.89 (t). . MS (FAB): 552 (M + H^+, 5), 433 (5), 379 (10), 316 (10), 288 (22), 185 (45), 149 (58). HRMS (FAB): calcd for $C_{21}H_{26}O_9N_7S$ 552.1513; found 552.1468.

2 – Amino – 4 - [5 - (6 – amino – purin – 9 - yl) - 3,4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylamino] – 4 – oxo - butyric acid (**55**). Compound **52** (20 mg, 0.029 mmol) was dissolved in 3 mL of 10% solution of TFA in water. Pd black (2 mg) was added and the reaction mixture was stirred in the atmosphere of hydrogen for 30 minutes. The catalyst was filtered off on the celite plug. Removal of solvent under reduced pressure afforded crude TFA salt **55**. Final purification was performed using Mitsubishi HP20SS gel. Sample was loaded in 5% TFA, washed with water and eluted with 0-50% MeOH in water. Y = 14 mg (85%). White, very hydroscopic solid. $[\alpha)_D^{27}$ = -4.3° (c = 0.47 in H_2O). IR (KBr): ν 3411, 3115, 2928, 1684, 1641, 1509, 1432, 1205, 1138, 976, 904, 839, 801, 724 cm^{-1}. ^1H NMR (300 MHz, H_2O): δ 8.49 (s, 1H), 8.43 (s, 1H), 6.17 (d, 1H, *J* = 4.3 Hz), 4.75 (m, 1H), 4.58 (t, 2H, *J* = 3.2 Hz), 4.52 (t, 1H, *J* = 5.0

Hz), 4.44 (m, 1H), 4.31 (t, 1H, *J* = 5.3 Hz) 3.09 (d, 2H, *J* = 5.7 Hz). ^{13}C NMR (75 MHz, H_2O) δ NO DATA COLLECTED DUE TO HIGH SALT CONTAMINATION. MS (ES FTICR): 462 (M + H^+, 80), 288 (15), 286 (60), 268 (15), 250 (100). HRMS (ESFTICR): calcd for $C_{14}H_{20}O_9N_7S$ 462.1043; found 462.1107.

4 - [5 - (6 – Amino – purin – 9 - yl) - 3,4 –dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylamino] – 4 – oxo - butyric acid (56). Compound **53** (20 mg, 0.037 mmol) was dissolved in 3 mL of 10% solution of TFA in water. Pd black (2 mg) was added and the reaction mixture was stirred in the atmosphere of hydrogen for 30 minutes. The catalyst was filtered off on the celite plug. Removal of solvent under reduced pressure afforded crude TFA salt **56**. Final purification was performed using Mitsubishi HP20SS gel. The sample was loaded in 5% TFA, washed with water and eluted with 0-50% MeOH in water. Y = 19 mg (90%). White, very hydroscopic solid. $[\alpha)_D^{27} = -3.4^o$ (c = 0.49 in H_2O). IR (KBr): ν 3405, 3126, 2928, 2631, 1684, 1624, 1431, 1204, 1138, 1048, 976, 892, 839, 801, 724 cm^{-1}. ^{1}H NMR (300 MHz, H_2O): δ 8.47 (s, 1H), 8.44 (s, 1H), 6.16 (d, 1H, *J* = 4.3 Hz), 4.75 (m, 1H), 4.59 (d, 2H, *J* = 2.8 Hz), 4.54 (t, 1H, *J* = 4.9 Hz), 4.45 (m, 1H), 2.61 (m, 4H). ^{13}C NMR (75 MHz, H_2O) δ NO DATA COLLECTED DUE TO HIGH SALT CONTAMINATION. MS (ES FTICR): 447 (M + H^+, 25), 268 (20), 250 (100). HRMS (ES FTICR): calcd for $C_{14}H_{19}O_9N_6S$ 447.0934; found 447.1002.

(3 – Amino - propionyl) - sulfamic acid 5 - (6 – amino – purin – 9 –y l) - 3,4 – dihydroxy – tetrahydro – furan – 2 - ylmethyl ester (57). Compound **54** (20 mg, 0.036 mmol) was dissolved in 3 mL of 10% solution of TFA in water. Pd black (2 mg) was added and the reaction mixture was stirred in the atmosphere of hydrogen for 30 minutes. The catalyst was filtered off on the celite plug. Removal of solvent under reduced pressure afforded crude TFA salt **57**. Final purification was performed using Mitsubishi HP20SS gel. The sample was loaded in 5% TFA, washed with water and eluted with 0-50% MeOH in water. Y = 17 mg (87%). White, very hydroscopic solid. $[\alpha)_D^{27}$ = -3.2° (c = 0.40 in H_2O). IR (KBr): ν 3397, 3170, 3109, 2928, 1679, 1632, 1481, 1435, 1205, 1135, 1049, 975, 902, 840, 802, 724 cm^{-1}. ^1H NMR (300 MHz, H_2O): δ 8.49 (s, 1H), 8.45 (s, 1H), 6.18 (d, 1H, *J* = 4.2 Hz), 4.75 (m, 1H), 4.63 (d, 2H, *J* = 3.1 Hz), 4.56 (t, 1H, *J* = 5.2 Hz), 4.45 (m, 1H),), 3.26 (t, 2H, *J* = 6.4 Hz), 2.84 (t, 2H, *J* = 6.3 Hz). ^{13}C NMR (75 MHz, H_2O) δ NO DATA COLLECTED DUE TO HIGH SALT CONTAMINATION. MS (ES FTICR): 418 ($M + H^+$, 10), 295 (10), 250 (100). HRMS (ES FTICR): calcd for $C_{13}H_{20}O_7N_7S$ 418.1145; found 418.1194.

4 - [6 - (6 – Benzoylamino – purin – 9 - yl) - 2, 2 –dimethyl – tetrahydro - furo[3, 4 - d][1, 3] dioxol – 4 - ylmethylaminosulfonylamino] – 2 – benzyloxycarbonylamino – 4 – oxo - butyric acid benzyl ester (**62**). A flame dried, nitrogen flushed 50 mL RBF was charged with N-succinimide ester **38c** (400 mg, 0.98 mMol) and amine **61** (409 mg, 0.98 mMol) in 20 mL of DMSO. DBU (146 μL, 0.98 mMol) was added and the reaction was stirred at room temperature overnight. Ethyl acetate (60 mL) of were added and the organic layer was washed with water (3 X 30 mL), dried over $MgSO_4$, filtered and solvent was removed under reduced pressure. Flash column chromatography (silica gel 80g, CH_2Cl_2/MeOH 98/2) yielded **62** as colorless oil (420 mg, 82%). $[\alpha)_D^{23}$ = -62.9° (c=0.38 in MeOH). IR (neat): ν 3410, 3329, 2932, 1705, 1662, 1610, 1455, 1247, 1212, 1094, 697 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.73 (s, 1H), 8.49 (s, 1H), 8.07 (d, 2H, *J* = 7.2 Hz), 7.59 (m, 3H), 7.27 (m, 10H), 6.18 (d, 1H, *J* = 3.2 Hz), 5.44 (dd, 1H, *J* = 6.3, 3.1 Hz), 5.12 (m, 2H), 5.05 (m, 2H), 4.97 (dd, 1H, *J* 6.6, 3.1 Hz), 4.65 (t, 1H, *J* = 6.2 Hz), 4.33 (dd, 1H, *J* = 8.4, 5.3 Hz), 3.63 (dd, 1H, *J* = 14.5, 6.0 Hz), 3.37 (dd, 1H, *J* = 13.9, 4.7 Hz), 2.86 (dd, 1H, *J* = 15.3, 6.1 Hz), 2.72 (dd, 1H, 15.1, 6.7 Hz), 1.58 (s, 3H), 1.34 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 172.73 (s), 172.31 (s), 168.07 (s), 158.28 (s), 153.34 (d), 152.76 (s), 151.41 (s), 145.21 (d), 138.04 (s), 137.10 (s), 134.93 (s), 133.94 (d), 129.77 (d), 129.51 (d), 129.47 (d), 129.45 (d), 129.18 (d), 129.07 (d), 128.97 (d), 128.79 (d), 125.63 (s), 115.77 (s), 92.32 (d), 86.10 (d), 84.66 (d), 83.32 (d), 68.13 (t), 67.72 (t), 52.50 (d), 42.15 (t), 38.71 (t), 27.52 (q), 25.57 (q). MS (EI): 812 ($M+H^+-CH_4$, 0.5), 240 (58), 180 (13), 136 (15), 130 (31), 105 (65), 99 (40), 91 (100). HRMS (EI): calcd for $C_{38}H_{36}N_8O_{11}S$ 812.2224; found 812.2239.

4 - [5 - (6 – Benzoylamino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethylaminosulfonylamino] – 2 – benzyloxycarbonylamino – 4 – oxo – butyric acid benzyl ester (63). Acetonide **62** (180 mg, 0.22 mMol) of were suspended in 7 mL of water. Concentrated TFA (7 mL) of were added and the reaction mixture was stirred for 1 hour. Solvent was removed under reduced pressure and crude material was chromatographed (silica gel, 10 g, CH_2Cl_2/MeOH 93/7) to afford 140 mg (82%) of diol **63** as colorless foam. $[\alpha]_D^{23}$ = -58.1° (c=0.42 in MeOH). IR (neat): ν 3406, 1696, 1649, 1612, 1456, 1252, 1200, 1060, 697 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.80 (s, 2H), 8.09 (d, 2H, *J* = 7.4 Hz), 7.68 (t, 1H, *J* =6.9 Hz), 7.57 (t, 2H, *J* = 8.0 Hz), 7.27 (m, 10H), 6.08 (d, 1H, *J* = 5.4 Hz), 5.13 (m, 2H), 5.05 (m, 2H), 4.82 (t, 1H, *J* = 5.3 Hz), 4.65 (t, 1H, *J* = 6.6 Hz), 4.20 (m, 2H), 3.76 (dd, 1H, *J* = 14.4, 6.3 Hz), 3.43 (dd, 1H, *J* = 14.1, 4.2 Hz), 2.87 (dd, 1H, *J* = 15.0, 5.8 Hz), 2.77 (dd, 1H, *J* = 15.5, 7.1 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 172.71 (s), 172.24 (s), 168.21 (s), 158.16 (s), 152.69 (d), 152.65 (s), 150.49 (s), 145.09 (d), 137.84 (s), 136.92 (s), 134.40 (s), 134.05 (d), 129.71 (d), 129.433 (d), 129.412 (d), 129.35 (d), 129.10 (d), 128.92 (d), 128.87 (d), 128.56 (d), 124.19 (s), 91.07 (d), 85.30 (d), 74.80 (d), 72.68 (d), 68.11 (t), 67.66 (t), 52.44 (d), 42.16 (t), 38.63 (t). MS (FAB): 772 (M+H$^+$-OH, 2), 240 (41), 91 (100). HRMS (FAB): calcd for $C_{36}H_{36}N_8O_{10}S$ 772.2275; found 772.2278.

2 – Amino – 4 - [5 - (6 – benzoylamino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethylaminosulfonylamino] – 4 - oxo - butyric acid (64). Intermediate **63** (130 mg, 0.16 mMol) was dissolved in 5 mL of 10% TFA in methanol. A catalytic amount of Pd black was added and reaction was stirred in the atmosphere of H_2 for 30 minutes. The reaction mixture was filtered through celite and solvent was evaporated. Crude material was purified using Mitsubishi HP20SS gel (load in 10%TFA in H_2O, washed with H_2O, eluted with increasing concentrations of MeOH in H_2O) to yield 75 mg (67%) of **64** as TFA salt. $[\alpha)_D^{23} = +6.5^o$ (c=0.4 in 5% HCl). IR (KBr): ν 3431, 1697, 1630, 1612, 1591, 1457, 1261, 1131, 1065, 704 cm^{-1}. ^{1}H NMR (300 MHz, D_2O): δ 8.44 (s, 1H), 8.32 (s, 1H), 7.61 (d, 2H, *J* = 8.6 Hz), 7.33 (t, 1H, *J* = 7.2 Hz), 7.19 (t, 2H, *J* = 7.7 Hz), 5.92 (d, 1H, *J* = 5.5 Hz), 4.69 (t, 1H, *J* = 5.4 Hz), 4.25 (t, 1H, *J* = 5.0 Hz), 4.17 (dd, 1H, *J* = 9.4, 4.6 Hz), 4.03 (t, 1H, *J* = 5.8 Hz), 3.63 (dd, 1H, *J* = 14.5, 5.8 Hz), 3.45 (dd, 1H, *J* = 14.1, 3.8 Hz), 2.95 (dd, 1H, *J* = 16.6, 4.8 Hz), 2.84 (dd, 1H, *J* = 16.7, 7.4 Hz). ^{13}C NMR (75 MHz, D_2O) δ 173.24 (s), 171.96 (s), 167.37 (s), 151.76 (s), 151.05 (d), 148.82 (s), 143.41 (d), 133.18 (s), 131.98 (d), 128.46 (d), 127.81 (d), 123.13 (s), 88.39 (d), 83.20 (d), 73.36 (d), 71.17 (d), 49.02 (d), 40.97 (t), 35.40 (t). MS (EI): 548 (M+H$^+$-OH, 20), 486 (42), 240 (100), 185 (56), 93 (67). HRMS (EI): calcd for $C_{21}H_{24}N_8O_8S$ 548.1437; found 548.1523.

2 – Amino – 4 - [5 - (6 – amino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethylaminosulfonylamino] – 4 – oxo - butyric acid (65) Benzamide **64** (65 mg, 0.096 mMol) was dissolved in 5 mL of concentrated NH_4OH and stirred overnight. After the reaction was completed, solvent was removed, the crude sample redissolved in 10% TFA and loaded on HP20SS column (washed with H_2O and sample eluted with increased concentration of MeOH in H_2O). Purification yielded 50 mg (90%) of **65** as TFA salt. $[\alpha)_D^{23} = -15.0^o$ (c=0.59 in H_2O). IR (KBr): ν 3388, 3132, 1672, 1506, 1409, 1399, 1198, 1133, 837, 800, 723 cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 8.41 (s, 2H), 6.05 (d, 1H, *J* = 4.9 Hz), 4.75 (t, 1H, *J* = 5.2 HZ), 4.32 (q, 2H, *J* = 5.4 Hz), 4.20 (q, 1H, *J* = 5.2 Hz), 3.59 (m, 2H)2.96 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ 171.00 (s), 170.92 (s), 150.05 (s), 148.28 (d), 144.53 (s), 143.20 (d), 118.30 (s), 88.95 (d), 83.10 (d), 73.72 (d), 70.95 (d), 49.52 (d), 40.73 (t), 34.42 (t).

3 - [5 - (6 – Amino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylaminocarbonyl] – pyrrolidine - 1, 2 - dicarboxylic acid 1 - benzyl ester (75a) and **3 - [5 - (6 – Amino – purin – 9 - yl) - 3,4 – dihydroxy – tetrahydro – furan – 2 -ylmethoxysulfonylaminocarbonyl] – pyrrolidine - 1, 2 - dicarboxylic acid 1 - benzyl ester (75b)**. A flame dried, argon flushed 100 mL RBF was charged with a mixture of diastereomeric carboxylic acids **73a** and **73b** (353 mg, 1.01 mMol) in 30 mL of freshly distilled MeCN. Catalytic amounts of DMAP and BtOH were added and the reaction was cooled to 0°C. EDCI (224 mg, 1.17 mMol) was added and the reaction was stirred at room temperature for 5 minutes followed by addition of neat sulfamate **44** (300 mg, 0.78 mMol). After 2 hours, solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel 100 g,

CH_2Cl_2/MeOH 9/1) to yield 280 mg (68% based on 80 mg of recovered **44**) of a mixture of **74a** and **74b**. This mixture was used for the next step. The mixture of **74a** and **74b** (120 mg, 0.17 mMol) was suspended in 5 mL of 50% solution of TFA in H_2O and stirred for 90 minutes. Solvent was removed and the crude mixture was dissolved in 10 mL of CH_2Cl_2. TFA (2 mL) of was added and reaction was stirred for another 90 minutes. Flash column chromatography (silica gel 5g, CH2Cl2/MeOH 8/2) yielded 80 mg (70%) of a mixture of **75a** and **75b** in 9:1 ratio based on ^1H NMR. Compounds **75a** and **75b** were separated using RP-HPLC on C-18 column. Elution with 0-20% gradient of MeCN in H_2O for 70 minutes, followed by 20-40% for 20 minutes, followed by 40-50% for 20 minutes results in isolation of **75a** (retention time 75 minutes). $[\alpha)_D^{23} = +23.2^o$ (c=0.4 in MeOH).IR (KBr): ν 3419, 3132, 2957, 2923, 1695, 1684, 1506, 1456, 1435, 1362, 1202, 1136, 980, 723 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.49 (s, 1H), 8.36 (s, 1H), 7.30 (m, 5H), 6.10 (t, 1H, $J = 4.2$ Hz), 5.09 (m, 2H), 4.58 (m, 4H), 4.43 (td, 1H, $J = 3.2, 1.5$ Hz), 4.32 (m, 1H), 3.56 (m, 2H), 3.18 (m, 1H), 2.23 (m, 1H), 2.12 (m, 1H). ^{13}C NMR (75 MHz, CD_3OD) inconclusive due to presence of rotamers. MS (EI): 622 (M + H^+, 33), 250 (45), 135 (9), 91 (100). HRMS (EI): calcd for $C_{24}H_{28}N_7O_{11}S$, 622.1567; found, 622.1567. Continuous elution yields **75b** (retention time 80 minutes). IR (KBr): ν 3431, 2957, 2856, 1695, 1684, 1631, 1459, 1428, 1384, 1362, 1201, 1136, 723, 573 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.45 (s, 1H), 8.35 (s, 1H), 7.34 (m, 5H), 6.10 (d, 1H, $J = 4.2$ Hz), 5.12 (m, 2H), 4.60 (m, 4H), 4.42 (m, 1H), 4.34 (m, 1H), 3.58 (m, 2H), 3.08 (m, 1H), 2.31 (m, 1H), 2.06 (m, 1H). ^{13}C NMR (75 MHz, CD_3OD) inconclusive due to presence of rotamers. MS (EI): 622 (M + H^+, 37), 277 (16), 185 (100), 93 (93). HRMS (EI): calcd for $C_{24}H_{28}N_7O_{11}S$, 622.1567; found, 622.1608.

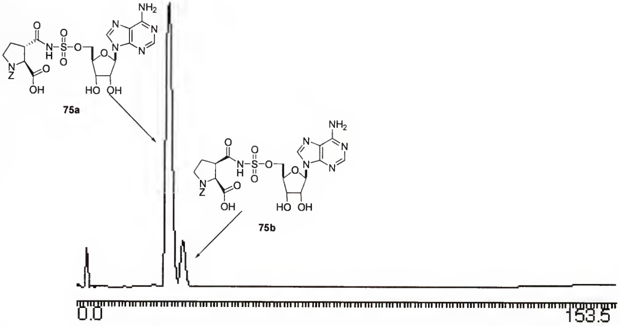


Figure 6-1. Chromatogram of **75a** and **75b** mixture

3 - [5 - (6 – Amino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylaminocarbonyl] – pyrrolidine – 2 - carboxylic acid (76). Intermediate **75a** (13 mg, 0.02 mMol) was dissolved in 5 mL of 20% TFA in MeOH. A catalytic amount of Pd black was added and the reaction was stirred in atmosphere of H_2 for 15 minutes. The reaction mixture was filtered through celite and solvent was evaporated. Purification on HP20SS gel (load in 10% TFA, washed with H_2O, eluted with increased gradient of MeOH in H_2O) yielded 10 mg (83%) of **76** as TFA salt. $[\alpha)_D^{23}$ = +18.2° (c=0.38 in H_2O). IR (KBr): ν 3418, 3115, 2957, 1693, 1684, 1652, 1633, 1384, 1203, 1138, 837, 801, 722 cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 8.52 (s, 1H), 8.43 (s, 1H), 6.17 (d, 1H, J = 3.2 Hz), 4.56 (m, 4H), 4.45 (m, 1H), 3.46 (m, 2H), 3.33 (m, 1H), 2.37 (m, 1H), 2.20 (m, 1H). ^{13}C NMR (75 MHz, D_2O) data not observed due to high salt concentration. MS (ES-FTICR): 488 (M + H^+). HRMS (ES-FTICR): calcd for $C_{16}H_{22}N_7O_9S$, 488.1194; found 488.1207.

3 - [5 - (6 – Amino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylaminocarbonyl] – pyrrolidine - 1, 2 - dicarboxylic acid dibenzyl ester (84a) and **3 - [5 - (6 – Amino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylaminocarbonyl] – pyrrolidine - 1, 2 - dicarboxylic acid dibenzyl ester (84b)**. A flame dried, argon flushed 100 mL RBF was charged with a carboxylic acid **82** (387 mg, 1.01 mMol) in 30 mL of freshly distilled MeCN. A catalytic amounts of DMAP and BtOH were added and the reaction was cooled to 0°C. EDCI (224 mg, 1.17 mMol) was added and reaction was stirred at room temperature for 5 minutes followed by addition of neat sulfamate **44** (300 mg, 0.78 mMol). After 2 hours solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel 100 g, CH_2Cl_2/MeOH 9/1) to yield 385 mg (66% based on 85 mg of recovered **44**) of **83**. Compound **83** (200 mg, 0.27 mMol) was suspended in 10 mL of 50% solution of TFA in H_2O and stirred for 90 minutes. Solvent was removed and crude was purified using flash column chromatography (silica gel 20 g, CH_2Cl_2/MeOH 8/2) to yield mixture of **84a** and **84b** in 9:1 ratio respectively (^{1}H NMR). Mixture of **84a** and **84b** were separated using RP-HPLC on C-18 column. Elution with 0-20% gradient of MeCN in H_2O for 70 minutes, followed by 20-40% for 20 minutes, followed by 40-50% for 20 minutes results in isolation of **84b** (retention time 83 minutes). IR (KBr): ν 3326, 3064, 2923, 1733, 1696, 1683, 1653, 1456, 1419, 1194, 1124, 976 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.45 (s, 1H), 8.32 (s, 1H), 7.25 (m, 10H), 6.06 (t, 1H, $J = 4.5$ Hz), 4.55 (m, 2H), 4.40 (m, 3H), 4.22 (m, 1H), 3.55 (m, 2H), 3.10 (m, 1H), 2.28 (m, 2H). ^{13}C NMR

(75 MHz, CD_3OD)) inconclusive due to presence of rotamers. MS (EI): 712 ($M + H^+$, 10), 250 (10), 136 (29), 91 (100). HRMS (EI): calcd for $C_{31}H_{34}N_7O_{11}S$, 712.2037; found 712.2052. Continuous elution yielded **84a** (retention time 94 minutes). $[\alpha]_D^{23} = +12.6^o$ (c=0.45 in MeOH). IR (KBr): ν 3338, 3117, 2925, 1736, 1701, 1683, 1498, 1456, 1419, 1360, 1197, 1137, 1003, 908 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ8.48 (s, 1H), 8.36 (s, 1H), 7.27 (m, 10H), 6.10 (d, 1H, $J = 4.6$ Hz), 5.11 (m, 4H), 4.84 (m, 1H), 4.66 (m, 2H), 4.59 (m, 2H), 4.46 (t, 1H, $J = 4.7$ Hz), 4.34 (m, 1H), 3.68 (m, 1H), 3.41 (m, 2H), 2.28 (m, 1H), 2.08 (m, 1H). ^{13}C NMR (75 MHz, CD_3OD)) inconclusive due to presence of rotamers. MS (EI): 712 ($M + H^+$, 43), 136 (57), 91 (100). HRMS (EI): calcd for $C_{31}H_{34}N_7O_{11}S$, 712.2037; found 712.2037.

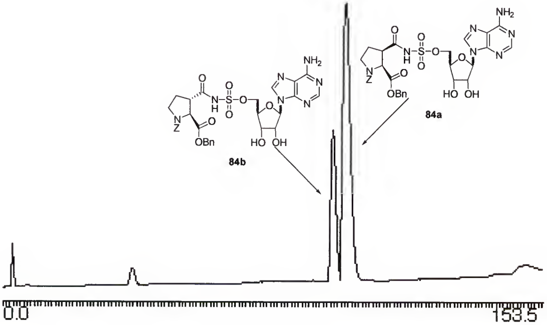


Figure 6-2. Chromatogram of **84a** and **84b** mixture

3 - [5 - (6 – Amino – purin – 9 - yl) - 3, 4 – dihydroxy – tetrahydro – furan – 2 - ylmethoxysulfonylaminocarbonyl] – pyrrolidine – 2 - carboxylic acid (85). Intermediate **84a** (15 mg, 0.02 mMol) was dissolved in 5 mL of 20% TFA in MeOH. A catalytic amount of Pd black was added and the reaction was stirred in atmosphere of H_2 for 15 minutes. The reaction mixture was filtered through celite and solvent was evaporated. Purification on HP20SS gel (load in 10% TFA, washed with H_2O, eluted with increased gradient of MeOH in H_2O) yielded 12 mg (99%) of **85** as TFA salt. $[\alpha)_D^{23} = +8.6^o$ (c=0.42 in H_2O). IR (KBr): ν 3421, 3115, 1695, 1684, 1652, 1634, 1204, 1140, 1047, 802, 723, 576 cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 8.54 (s, 1H), 8.42 (s, 1H), 6.17 (d, 1H, *J* = 4.8 Hz), 4.51 (m, 1H), 4.40 (m, 4H), 3.50 (m, 4H), 2.46 (m, 1H), 2.30 (m, 1H). ^{13}C NMR (75 MHz, D_2O) data not observed due to high salt concentration. MS (ES-FTICR): 488 (M + H^+). HRMS (ES-FTICR): calcd for $C_{16}H_{22}N_7O_9S$, 488.1194; found 488.1208.

Sulfamic acid 2 - (4 – nitro - phenylamino) - ethyl ester (90). A flame dried, nitrogen flushed 250 mL RBF was charged with sodium hydride (0.29g, 7.2 mmol) in 50 mL of freshly distilled THF and cooled to 0°C in the ice bath. Alcohol **86** (1g, 5.5 mmol) in 20 mL of THF was added drop wise. After 15 minutes the reaction was warmed to room temperature and allowed to stir for another 45 minutes and then cooled to 0°C. Neat sulfamoyl chloride (1.25g, 11 mmol) was added in portions and reaction was stirred for 30 minutes. Excess sodium hydride was quenched with methanol. The reaction mixture was adsorbed on silica gel and solvent removed under reduced pressure. The resulted material was subjected to purification using flash column chromatography (dry load, silica gel, MeOH/CH2Cl2 3/97). Fractions containing product were combined and solvent was removed. Pure sulfamate was precipitated using dichloromethane. Y = 1.05g (73%). Yellow solid material. m.p. = 124 – 126 °C. IR (neat): ν 3615, 3376, 3284, 1619, 1574, 1511, 1359, 1181, 1000, 930, 775, 552 cm^{-1}. ^{1}H NMR (300 MHz, DMSO + D_2O): δ 7.98 (d, 2H, *J* = 9.3 Hz), 6.68 (d, 2H, *J* = 9.3 Hz), 4.15 (t, 2H, *J* = 5.2 Hz), 3.50 (t, 2H, *J* = 5.2 Hz). ^{13}C NMR (75 MHz, DMSO) δ 154.21 (s), 136.07 (s), 126.20 (d), 110.97 (d), 67.08 (t), 41.42 (t). MS (FAB): 262 (M + H$^+$, 100), 246 (17), 107 (37), 97 (64), 85 (75). HRMS (FAB): calcd for $C_8H_{12}N_3O_5S$, 262.0498; found, 262.0526.

Sulfamic acid 2 - (2 – nitro - phenylamino) - ethyl ester (91). A flame dried, nitrogen flushed 250 mL RBF was charged with sodium hydride (0.29g, 7.2 mmol) in 50 mL of freshly distilled THF and cooled to 0°C in the ice bath. Alcohol **87** (1g, 5.5 mmol) in 20 mL of THF was added drop wise. After 15 minutes the reaction was warmed to room temperature and allowed to stir for another 45 minutes and then cooled to 0°C. Neat sulfamoyl chloride (1.25g, 11 mmol) was added in portions and the reaction was stirred for 30 minutes. Excess sodium hydride was quenched with methanol. The reaction mixture was adsorbed on silica gel and solvent removed under reduced pressure. The resulted material was subjected to purification using flash column chromatography (dry load, silica gel, MeOH/CH2Cl2 3/97). Fractions containing product were combined and solvent was removed. Pure sulfamate was precipitated using dichloromethane. Y = 1.15g (80%). Orange solid material. m.p. = 94 - 96°C. IR (neat): ν 3606, 3378, 3287, 1620, 1574, 1512, 1361, 1181, 999, 930, 776, 552 cm^{-1}. ^{1}H NMR (300 MHz, DMSO + D_2O): δ 8.05 (dd, 1H, *J* = 8.7, 1.3 Hz), 7.52 (m, 1H), 7.06 (d, 1H, *J* = 8.5 Hz), 6.71 (t, 1H, *J* = 7.8 Hz), 4.23 (t, 2H, *J* = 5.3 Hz), 3.69 (t, 2H, *J* = 5.2 Hz). ^{13}C NMR (75 MHz, DMSO) δ 145.86 (s), 136.70 (d), 131.39 (s), 126.35 (d), 115.73 (d), 114.48 (d), 67.10 (t), 41.59 (t).

MS (FAB): 262 (M + H^+,100), 161 (61), 149 (55), 107 (44), 97 (52), 83 (73). HRMS (FAB): calcd for $C_8H_{12}N_3O_5S$, 262.0498; found, 262.0497.

Sulfamic acid 2 - (2,4 – dinitro - phenylamino) - ethyl ester (92). A flame dried, nitrogen flushed 250 mL RBF was charged with sodium hydride (0.23g, 5.7 mmol) in 50 mL of freshly distilled THF and cooled to 0°C in the ice bath. Alcohol **86** (1g, 4.4 mmol) in 20 mL of THF was added drop wise. After 15 minutes the reaction was warmed to room temperature and allowed to stir for another 45 minutes and then cooled to 0°C. Neat sulfamoyl chloride (1.0g, 9 mmol) was added in portions and the reaction was stirred for 30 minutes. Excess sodium hydride was quenched with methanol. The reaction mixture was adsorbed on silica gel and solvent removed under reduced pressure. The resulted material was subjected to purification using flash column chromatography (dry load, silica gel, MeOH/CH2Cl2 3/97). Fractions containing product were combined and solvent was removed. Pure sulfamate was precipitated using dichloromethane. Y = 1.0g (74%). Light orange solid. m.p. = 128 – 130 °C. IR (neat): ν 3590, 3357, 3289, 1620, 1590, 1338, 1181, 923, 744 cm^{-1}. ^1H NMR (300 MHz, DMSO + D_2O): δ 8.80 (d, 1H, *J* = 2.7 Hz), 8.21 (dd, 1H, *J* = 9.8, 2.7 Hz), 7.20 (d, 1H, *J* = 9.8 Hz), 4.25 (t, 2H, *J* = 5.1 Hz), 3.78 (t, 2H, *J* = 5.1 Hz). ^{13}C NMR (75 MHz, DMSO) δ 148.29 (s), 135.15 (s), 130.07 (s), 129.97 (d), 123.82 (d), 115.45 (d), 66.95 (t), 41.85 (t). MS (FAB): 307 (M + H^+, 10), 273 (5), 244 (5), 183 (7), 107 (100). HRMS (FAB): calcd for $C_8H_{12}O_7N_4S$ 307.0348; found, 307.0343.

Sulfamic acid 4 - (2, 4 – dinitro - phenylamino) - butyl ester (93). A flame dried, nitrogen flushed 250 mL RBF was charged with sodium hydride (0.31g, 7.7 mmol) in 50 mL of freshly distilled THF and cooled to 0°C in the ice bath. Alcohol **89** (1.5 g, 5.9

mmol) in 20 mL of THF was added drop wise. After 15 minutes the reaction was warmed to room temperature and allowed to stir for another 45 minutes and then cooled to 0°C. Neat sulfamoyl chloride (1.4 g, 11.8 mmol) was added in portions and reaction was stirred for 30 minutes. Excess sodium hydride was quenched with methanol. The reaction mixture was adsorbed on silica gel and solvent removed under reduced pressure. The resulted material was subjected to purification using flash column chromatography (dry load, silica gel, MeOH/CH2Cl2 3/97). Fractions containing product were combined and solvent was removed. Pure sulfamate **93** was precipitated using dichloromethane. Y = 1.6 g (82%). IR (neat): ν 3372, 1615, 1364, 1333, 1173, 992, 919, 769 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 9.02 (d, 1H, *J* = 2.6 Hz), 8.28 (dd, 1H, *J* = 9.7, 2.9 Hz), 7.18 (d, 1H, *J* = 9.5 Hz), 4.20 (t, 2H, *J* = 5.9 Hz), 3.55 (t, 2H, *J* = 6.5 Hz), 1.87 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 149.67 (s), 143.51 (s), 137.28 (s), 131.06 (d), 124.75 (d), 115.74 (d), 70.47 (t), 43.66 (t), 27.30 (t), 26.09 (t). MS (EI): 334 (M^+, 10), 237 (26), 220 (100), 190 (85), 189 (60), 149 (32), 76 (18). HRMS (EI): calcd for $C_{10}H_{14}O_7N_4S$ 334.0583; found, 334.0607.

2 – Benzyloxycarbonylamino – 4 - [2 - (2 - nitro phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (**94**). A flame dried, nitrogen flushed 25 mL RBF was charged with 410mg (1.15 mmol) of N-Cbz-α-benzyl aspartic acid **38a** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 220mg (1.15 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0. 77 mmol) of adenosine sulfamate **90** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified

using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 310mg. Light orange semi - solid material. $[\alpha)_D^{28}$ = +2.6° (c = 0.22 in MeOH). IR (neat): ν 3379, 1710, 1715, 1618, 1573, 1512, 1263, 1150, 1043, 742 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.07 (d, 1H, *J* = 8.5 Hz), 7.45 (t, 1H, *J* = 7.2 Hz), 7.30 (m, 10H), 6.97 (d, 1H, *J* = 8.8 Hz), 6.64 (t, 1H, *J* = 7.6 Hz), 5.04 (s, 2H), 4.59 (s, 2H), 4.59 (m, 1H), 4.24 (t, 2H, *J* = 5.4 Hz), 3.58 (t, 2H, *J* = 5.3 Hz), 2.70 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 179.02 (s), 174.29 (s), 158.43 (s), 146.41 (s), 142.68 (s), 138.02 (s), 137.61(s), 133.24 (d), 129.46 (d), 129.40 (d), 129.05 (d), 128.85 (d), 128.30 (d), 128.02 (d), 127.57 (d), 116.70 (d), 115.26 (d), 68.12 (t), 67.78 (t), 65.27 (t), 52.70 (d), 43.06 (t), 41.65 (t). MS (ESI): 645 (M - H^+ + $2Na^+$, 40), 623 (M + Na^+, 100), 547 (50), 351 (20). HRMS (ESI): calcd for $C_{27}H_{28}O_{10}N_4SNa$ 623.1419; found 623.1443.

4 - [2 - (2 – Nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (**95**). A flame dried, nitrogen flushed 25 mL RBF was charged with 240mg (1.15 mmol) of monobenzyl succinate **39** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 220mg (1.15 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0.77 mmol) of adenosine sulfamate **90** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 210mg (60%). Light orange semi – solid. IR (neat): ν 3377, 1728, 1619, 1573, 1512, 1354, 1263, 1147, 742 cm^{-1}. ^1H NMR (300 MHz, DMSO): δ 8.07 (d, 1H, *J* = 8.8 Hz), 7.53 (t, 1H, *J* =

7.4 Hz), 7.36 (m, 5H), 7.09 (d, 1H, *J* = 8.5 Hz), 6.91 (t, 1H, *J* = 7.8 Hz), 5.05 (s, 2H), 4.08 (t, 2H, *J* = 5.4 Hz), 3.57 (t, 2H, *J* = 5.4 Hz), 2.47 (t, 2H, *J* = 6.7 Hz), 2.31 (t, 2H, *J* = 6.8 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.23 (s), 175.06 (s), 146.55 (s), 137.74 (s), 137.62 (s), 133.43 (d), 129.61 (d), 129.16 (d), 129.12 (d), 127.65 (d), 116.72 (d), 115.37(d), 68.00 (t), 67.35 (t), 43.09 (t), 34.63 (t), 31.12 (t). MS (FAB): 496 ($M - H^+ + 2Na^+$, 17), 474 ($M + Na^+$, 23), 456 (12), 341 (5), 299 (28), 230 (17), 107 (40). HRMS (FAB): calcd for $C_{19}H_{20}N_3O_8SNa_2$ 496.0767; found 496.0795.

{3 - [2 - (2 – Nitro - phenylamino) - ethoxysulfonylamino] – 3 – oxo - propyl} - carbamic acid benzyl ester (96). A flame dried, nitrogen flushed 25 mL RBF was charged with 258mg (1.15 mmol) of N-Cbz-β-alanine **40** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 220mg (1.15 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0.77 mmol) of adenosine sulfamate **90** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, $MeOH/CH_2Cl_2$ 3/97). An analytical sample was prepared using preparative scale TLC. Y = 200mg (56%). Light orange semi – solid. IR (neat): ν 3369, 1673, 1596, 1349, 1142 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.09 (dd, 1H, *J* = 8.5, 1.2 Hz), 7.46 (dt, 1H, *J* = 7.3, 1.2 Hz), 7.30 (m, 5H), 7.00 (d, 1H, *J* = 8.5 Hz), 6.65 (m, 1H), 5.02 (s, 2H), 4.25 (t, 2H, *J* = 5.6 Hz), 3.62 (t, 2H, *J* = 5.6 Hz), 3.34 (t, 2H, *J* = 6.4 Hz), 2.36 (t, 2H, *J* = 6.4 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.07 (s), 158.75 (s), 146.48 (s), 138.41 (s), 137.60 (s), 133.40 (d), 129.54 (d), 129.02 (d), 128.88 (d), 127.64 (d), 116.72 (d), 115.32 (d), 68.07 (t), 67.46 (t), 43.13 (t), 40.04 (t), 38.82 (t). MS (FAB): 511 ($M - H^+ + 2Na^+$, 25), 489 (M

+ Na$^+$, 36), 456 (10), 341 (5), 245 (22), 91 (35). HRMS (FAB): calcd for $C_{19}H_{22}O_8N_4SNa$ 489.1056; found 489.1036.

2 – Benzyloxycarbonylamino – 4 - [2 - (4 – nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (97). A flame dried, nitrogen flushed 25 mL RBF was charged with 410mg (1.15 mmol) of N-Cbz-α-benzyl aspartic acid **38a** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 220mg (1.15 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0. 77 mmol) of adenosine sulfamate **91** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 251mg (55%). Yellow semi – solid.

$[\alpha]_D^{28} = +8.4^\circ$ (c = 0.20 in MeOH). IR (neat): ν 3377, 1727, 1715, 1602, 1324, 1309, 1287, 1142 cm^{-1}. . ^{1}H NMR (300 MHz, CD_3OD): δ 7.98 (d, 2H, J = 9.1 Hz), 7.28 (m, 10H), 6.60 (d, 2H, J = 9.1 Hz), 5.00 - 5.20 (m, 4H), 4.50 (m, 1H), 4.18 (t, 2H, J = 5.1 Hz), 3.40 (t, 2H, J = 5.1 Hz), 2.76 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 185.23 (s), 179.17 (s), 173.62 (s), 155.59 (s), 138.37 (s), 137.19 (s), 130.02 (s), 129.62 (d), 129.55 (d), 129.24 (d), 129.02 (d), 128.87 (d), 128.07 (d), 127.38 (d), 112.20 (d), 68.27 (t), 68.17 (t), 67.83 (t), 52.88 (d), 43.17 (t), 41.60 (t). MS (FAB): 645 (M - H$^+$ + 2Na$^+$, 10), 623 (M + Na$^+$, 13), 402 (5). HRMS (FAB): calcd for $C_{27}H_{28}O_{10}N_4SNa$ 623.1424; found 623.1426.

4 - [2 - (4 – Nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (**98**). A flame dried, nitrogen flushed 25 mL RBF was charged with 240mg (1.15 mmol) of monobenzyl succinate **39** in 5 mL of freshly distilled acetonitrile and cooled to 0^oC. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 220mg (1.15 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0^oC. 200 mg (0.77 mmol) of adenosine sulfamate **91** in 5 mL of acetonitrile was added at 0^oC. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, $MeOH/CH_2Cl_2$ 3/97). An analytical sample was prepared using preparative scale TLC. Y = 250mg (72%). Yellow semi - solid. IR (neat): ν cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 7.98 (d, 2H, $J = 9.3$ Hz), 7.30 (m, 5H), 6.62 (d, 2H, $J = 9.1$ Hz), 5.05 (s, 2H), 4.20 (t, 2H, $J = 5.4$ Hz), 3.45 (t, 2H, $J = 5.3$ Hz), 2.62 (t, 2H, $J = 6.2$ Hz), 2.56 (t, 2H, $J = 6.2$ Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.48 (s), 175.02 (s), 155.60 (s), 138.28 (s), 137.56 (s), 129.57 (d), 129.14 (d), 129.04 (d), 127.36 (d), 112.17 (d), 68.17 (t), 67.36 (t), 43.16 (t), 34.64 (t), 31.07 (t). MS (FAB): 452 ($M + H^+$, 30), 415 (5), 300 (7), 208 (95), 132 (50), 91 (100). HRMS (FAB): calcd for $C_{19}H_{22}O_8N_3S$ 452.1128; found 452.1122.

{3 - [2 - (4 – Nitro - phenylamino) - ethoxysulfonylamino] – 3 - oxo-propyl} - carbamic acid benzyl ester (**99**). A flame dried, nitrogen flushed 25 mL RBF was charged with 258mg (1.15 mmol) of N-Cbz-β-alanine **40** in 5 mL of freshly distilled acetonitrile and cooled to 0^oC. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 220mg (1.15 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0^oC. 200 mg (0.77 mmol) of adenosine sulfamate **91** in 5 mL of acetonitrile was added at 0^oC. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 180mg (56%). Orange semi – solid. IR (neat): ν cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.00 (d, 2H, *J* = 9.1 Hz), 7.30 (m, 5H), 6.62 (d, 2H, *J* = 9.1 Hz), 5.02 (s, 2H), 4.20 (t, 2H, *J* = 5.4 Hz), 3.46 (t, 2H, *J* = 5.3 Hz), 3.34 (t, 2H, *J* = 6.7 Hz), 2.38 (t, 2H, *J* = 6.6 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.24 (s), 158.74 (s), 155.64 (s), 138.41 (s), 138.35 (s), 129.54 (d), 129.02 (d), 128.86 (d), 127.36 (d), 112.19 (d), 68.22 (t), 67.49 (t), 43.24 (t), 40.12 (t), 38.87 (t). MS (FAB): 511 ($M - H^+ + 2Na^+$, 5), 489 ($M + Na^+$, 16), 460 (5), 369 (3), 245 (16). HRMS (FAB): calcd for $C_{19}H_{22}O_8N_4SNa$ 489.1056; found 489.1056.

2 – Benzyloxycarbonylamino – 4 - [2 - (2,4 – dinitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid benzyl ester (100). A flame dried, nitrogen flushed 25 mL RBF was charged with 357mg (1.0 mmol) of N-Cbz-α-benzyl aspartic acid **38a** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 192mg (1.0 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0. 66 mmol) of adenosine sulfamate **92** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 310mg (72%). Yellow semi – solid.

$[\alpha)_D^{28}$ = +3.3° (c = 0.23 in MeOH). IR (neat): ν 3418, 1715, 1620, 1589, 1338, 1309, 1150, 744 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.92 (d, 1H, *J* = 2.5 Hz), 8.22 (dd, 1H, *J* = 9.5, 2.5 Hz), 7.28 (m, 10H), 7.17 (d, 1H, *J* = 9.6 Hz), 5.04 (s, 2H), 4.58 (s, 2H), 4.36

(m, 1H), 4.27 (t, 2H, *J* = 5.4 Hz), 3.74 (m, 2H), 2.75 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 178.97 (s), 174.27 (s), 158.46 (s), 149.78 (s), 142.73 (s), 138.10 (s), 137.22 (s), 131.78 (s), 131.15 (d), 129.52 (d), 129.42 (d), 129.05 (d), 128.81 (d), 128.34 (d), 128.06 (d), 124.69 (d), 116.13 (d), 67.99 (t), 67.78 (t), 65.32 (t), 52.77 (d), 43.65 (t), 41.68 (t). MS (FAB): 646 (M + H$^+$, 20), 513 (4), 391 (5), 358 (5), 255 (7), 173 (10), 155 (35). HRMS (FAB): calcd for $C_{27}H_{28}O_{12}N_5S$ 646.1455; found 646.1444.

4 - [2 - (2,4 - Dinitro-phenylamino) - ethoxysulfonylamino] – 4 – oxo – butyric acid benzyl ester (101). A flame dried, nitrogen flushed 25 mL RBF was charged with 209mg (1.0 mmol) of monobenzyl succinate **39** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 192mg (1.0 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0.66 mmol) of adenosine sulfamate **92** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 201mg (61%). Yellow semi – solid. IR (neat): ν 3354, 1727, 1620, 1589, 1524, 1338, 1310, 1145, 744 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.87 (d, 1H, *J* = 2.3 Hz), 8.20 (dd, 1H, *J* = 9.6, 2.6 Hz), 7.28 (m, 5H), 7.18 (d, 1H, *J* = 9.6 Hz), 5.05 (s, 2H), 4.28 (t, 2H, *J* = 5.1 Hz), 3.75 (t, 2H, *J* = 5.1 Hz), 2.60 (t, 2H, *J* = 6.2 Hz), 2.52 (t, 2H, *J* = 6.2 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.31 (s), 174.98 (s), 149.70 (s), 137.60 (s), 137.08 (s), 131.64 (s), 131.10 (d), 129.52 (d), 129.05 (d), 128.92 (d), 124.62 (d), 116.13 (d), 67.86 (t), 67.27 (t), 43.62 (t), 34.62 (t), 31.04 (t). MS (FAB): 497 (M + H$^+$, 10), 393 (23), 301 (44), 209 (100), 91 (89). HRMS (FAB): calcd for $C_{19}H_{21}O_{10}N_4S$ 497.0978; found 497.1046.

{3 - [2 - (2,4 – Dinitro - phenylamino) - ethoxysulfonylamino] – 3 – oxo - propyl} - carbamic acid benzyl ester (102). A flame dried, nitrogen flushed 25 mL RBF was charged with 224mg (1.0 mmol) of N-Cbz-β-alanine **40** in 5 mL of freshly distilled acetonitrile and cooled to 0°C. DMAP (cat.), hydroxybenzotriazole (cat.) followed by 192mg (1.0 mmol) of EDCI were added. The reaction mixture was stirred for 5 minutes at 0°C. 200 mg (0.66 mmol) of adenosine sulfamate **92** in 5 mL of acetonitrile was added at 0°C. The reaction was stirred overnight. Solvent was removed under reduced pressure and crude material was purified using flash column chromatography (silica gel, MeOH/CH_2Cl_2 3/97). An analytical sample was prepared using preparative scale TLC. Y = 190mg (56%). Yellow semi – solid. IR (neat): ν 3356, 1703, 1619, 1587, 1523, 1338, 1308, 1136 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.91 (d, 1H, $J = 2.3$ Hz), 8.22 (dd, 1H, $J = 9.6, 2.6$ Hz), 7.28 (m, 5H), 7.17 (d, 1H, $J = 9.6$ Hz), 5.01 (s, 2H), 4.28 (t, 2H, $J = 5.3$ Hz), 3.77 (t, 2H, $J = 5.2$ Hz), 3.34 (t, 2H, $J = 6.6$ Hz), 2.36 (t, 2H, $J = 6.6$ Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 181.14 (s), 158.70 (s), 149.75 (s), 138.34 (s), 137.19 (s), 131.77 (s), 131.12 (d), 129.50 (d), 128.98 (d), 128.78 (d), 124.68 (d), 116.09 (d), 67.93 (t), 67.43 (t), 43.66 (t), 40.07 (t), 38.82 (t). MS (FAB): 512 ($M + H^+$, 45), 460 (3), 357 (5), 338 (5), 255 (15), 185 (11), 173 (35), 91 (62). HRMS (FAB): calcd for $C_{19}H_{22}O_{10}N_5S$ 512.1087; found 512.1077.

(3 – Amino - propionyl) - sulfamic acid 2 - (2 – nitro - phenylamino) - ethyl ester (103). A flame dried 10 mL RBF was charged with 22 mg (0.047 mmol) of carbamate **96** in 3 mL of freshly distilled acetonitrile. Trimethylsilyliodide (0.24 mmol) was added from a syringe at room temperature. The reaction mixture was stirred for 10 minutes followed by removal of the solvent under reduced pressure. Preparative TLC purification of the crude mixture afforded pure amine **103**. Y= 12 mg (77%). Yellow semi – solid material. IR (neat): ν 3444, 1620, 1573, 1511, 1356, 1263, 1150, 1019, 844 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.12 (d, 1H, *J* = 8.5 Hz), 7.49 (dt, 1H, *J* = 8.5, 1.5 Hz), 7.06 (d, 1H, *J* = 8.5 Hz), 6.68 (t, 1H, *J* = 7.6 Hz), 4.30 (t, 2H, *J* = 5.4 Hz), 3.68 (t, 2H, *J* = 5.4 Hz), 3.00 (t, 2H, *J* = 6.1 Hz), 2.44 (t, 2H, *J* = 6.2 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 179.87 (s), 146.57 (s), 137.61 (s), 133.51 (d), 127.68 (d), 116.73 (d), 115.40 (d), 68.17 (t), 43.23 (t), 38.37 (t), 38.30 (t). 333 (5), 115 (100). HRMS (FAB): calcd for $C_{11}H_{17}O_6N_4S$ 333.0869; found 333.0858.

(3 – Amino - propionyl) - sulfamic acid 2 - (4 – nitro - phenylamino) - ethyl ester (104). A flame dried 10 mL RBF was charged with 35 mg (0.075 mmol) of carbamate **99** in 3 mL of freshly distilled acetonitrile. Trimethylsilyliodide (0.24 mmol) was added from a syringe at room temperature. The reaction mixture was stirred for 10 minutes followed by removal of the solvent under reduced pressure. Preparative TLC purification of the crude mixture afforded pure amine **104**. Y = 18 mg (72%). Yellow semi – solid material. IR (neat): ν 3479, 1605, 1469, 1310, 1148, 1117, 836 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.02 (d, 2H, *J* = 9.4 Hz), 6.68 (d, 2H, *J* = 9.4 Hz), 4.23 (t, 2H, *J* = 5.4 Hz), 3.52 (t, 2H, *J* = 5.6 Hz), 3.01 (t, 2H, *J* = 6.3 Hz), 2.46 (t, 2H, *J* = 6.3 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 179.86 (s), 155.79 (s), 138.62 (s), 127.38 (d), 112.29 (d), 68.34 (t), 43.37 (t), 38.37 (t), 38.30 (t). MS (FAB): 333 (10), 231 (8), 207 (65), 115 (90). HRMS (FAB): calcd for $C_{11}H_{17}O_6N_4S$ 333.0869; found 333.0858.

(3 – Amino - propionyl) - sulfamic acid 2 - (2,4 – dinitro - phenylamino) - ethyl ester (105). A flame dried 10 mL RBF was charged with 25 mg (0.049 mmol) of carbamate **102** in 3 mL of freshly distilled acetonitrile. Trimethylsilyliodide (0.24 mmol) was added from a syringe at room temperature. The reaction mixture was stirred for 10 minutes followed by removal of the solvent under reduced pressure. Preparative TLC purification of the crude mixture afforded pure amine **105**. Y = 14 mg (75%). Yellow semi – solid material. IR (neat): ν 3467, 1620, 1523, 1340, 1312, 1149, 835 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 9.03 (d, 1H, *J* = 2.6 Hz), 8.28 (dd, 1H, *J* = 9.6, 2.6 Hz), 7.27 (d, 1H, *J* = 9.6 Hz), 4.32 (t, 2H, *J* = 5.3 Hz), 3.82 (t, 2H, *J* = 5.3 Hz), 2.98 (t, 2H, *J* = 5.9 Hz), 2.43 (t, 2H, *J* = 6.2 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 179.95 (s), 149.95 (s), 137.91 (s), 132.06 (s), 131.15 (d), 124.78 (d), 116.21 (d), 68.02 (t), 43.75 (t), 38.73 (t), 38.42 (t). MS (FAB): 378 (12), 373 (5), 299 (7), 231 (10), 207 (66), 130 (6), 115 (100). HRMS (FAB): calcd for $C_{11}H_{15}O_8N_5S$ 378.0720; found 378.0708.

4 - [2 - (2 – Nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid (106). Benzyl ester **95** (80 mg, 0.18 mMol) was dissolved in 4 mL of 50% THF mixture in H_2O. KOH (15 mg, 0.27 mMol) was added and the reaction was stirred for 2 hours (TLC showed no starting material left). The reaction mixture was acidified with 1N HCl and solvent was evaporated. Flash column chromatography (silica gel 5 g, CH_2Cl_2/MeOH 9/1) yielded desired carboxylic acid **106** (53 mg, 82%).(neat): ν 3421, 1689, 1630, 1575, 1510, 1410, 1358, 1210, 1146, 915, 847, 800 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.12 (dd, 1H, *J* = 8.5, 1.5 Hz), 7.49 (m, 1H), 7.06 (dd, 1H, *J* = 8.8, 1.2 Hz), 6.68 (m, 1H), 4.28 (t, 2H, *J* = 5.6 Hz), 3.68 (t, 2H, *J* = 5.6 Hz), 2.46 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 182.42 (s), 180.40 (s), 146.40 (s), 137.49 (d), 133.27 (s), 127.50 (d), 116.57 (d), 115.19 (d), 67.99 (t), 43.03 (t), 36.07 (t), 33.59 (t). MS (ES-FTICR): 428 (M-2H+3Na$^+$), 406 (M-H+2Na$^+$), 384 (M+Na$^+$). HRMS (ES-FTICR): calcd for $C_{12}H_{15}O_8N_3SNa$ 384.0478; found 384.0439.

4 - [2 - (4 – Nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid (107). Benzyl ester **98** (70 mg, 0.16 mMol) was dissolved in 4 mL of 50% THF mixture in H_2O. KOH (13 mg, 0.23 mMol) was added and the reaction was stirred for 2 hours (TLC showed no starting material left). The reaction mixture was acidified with 1N HCl and solvent was evaporated. Flash column chromatography (silica gel 5 g, CH_2Cl_2/MeOH 9/1) yielded desired carboxylic acid **107** (47 mg, 84%).(neat): ν 3390, 1680, 1608, 1507, 1469, 1389, 1340, 1203, 1142, 836 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.03 (d, 2H, *J* = 9.2 Hz), 6.69 (d, 2H, *J* = 9.2 Hz), 4.22 (t, 2H, *J* = 5.4 Hz), 3.50 (t, 2H, *J* = 5.4 Hz), 2.47 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 183.43 (s), 182.13 (s), 155.82 (s), 138.44 (s), 127.39 (d), 112.20 (d), 68.17 (t), 43.35 (t), 37.01 (t), 35.10 (t). MS (ES-FTICR): 449 ($M-3H+4Na^+$), 428 ($M-2H+3Na^+$), 406 ($M-H+2Na^+$). HRMS (ES-FTICR): calcd for $C_{12}H_{14}O_8N_3SNa_2$ 406.0296; found 406.0257.

4 - [2 - (2, 4 – Dinitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid (108). Benzyl ester **101** (100 mg, 0.2 mMol) was dissolved in 4 mL of 50% THF mixture in H_2O. KOH (17 mg, 0.30 mMol) was added and the reaction was stirred for 2 hours (TLC showed no starting material left). The reaction mixture was acidified with 1N HCl and solvent was evaporated. Flash column chromatography (silica gel 5 g, CH_2Cl_2/MeOH 9/1) yielded desired carboxylic acid **108** (73 mg, 90%).(neat): ν 3440, 1689, 1620, 1432, 1344, 1319, 1211, 1140, 841, cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.95 (d, 1H, *J* = 2.9 Hz), 8.28 (dd, 1H, *J* = 9.6, 2.6 Hz), 7.27 (d, 1H, *J* = 9.7 Hz), 4.31 (t, 2H, *J* = 5.4 Hz), 3.81 (t, 2H, *J* = 5.3 Hz), 2.45 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 182.73 (s), 180.81 (s), 149.74 (s), 137.10 (s), 131.72 (s), 131.02 (d), 124.55 (d), 116.04 (d), 67.84 (t), 43.58 (t), 36.31 (t), 33.95 (t). MS

(ES-FTICR): 472 (M-2H+3Na$^+$), 451 (M-H+2Na$^+$), 429 (M+Na$^+$). HRMS (ES-FTICR): calcd for $C_{12}H_{14}O_{10}N_4SNa$ 429.0328; found 429.0279.

2 – *tert* – Butoxycarbonylamino – 4 - [2 - (4 – nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid *tert* - butyl ester (109). A flame dried, nitrogen flushed 100 mL RBF was charged with aspartate **38d** (498 mg, 1.72 mMol) in 30 mL of freshly distilled MeCN. Catalytic amounts of DMAP and BtOH was added and reaction mixture was cooled to 0°C. EDCI (330 mg, 1.72 mMol) was added and the reaction was stirred at room temperature for 30 minutes. After cooling back to 0°C, sulfamate **90** (300 mg, 1.15 mMol) was added and the reaction was stirred to room temperature overnight. The solvent was evaporated, flash column chromatography (silica gel 75g, CH_2Cl_2/MeOH 95/5) yielded intermediate **109** (514 mg, 84%). $[\alpha)_D^{25} = -7.2^\circ$ (c = 0.3 in MeOH). IR (neat): ν 3521, 3378, 2979, 2934, 1721 (broad), 1692, 1602, 1369, 1324, 1310, 1288, 1152, 1112, 838 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.02 (d, 2H, J = 10.4 Hz), 6.68 (d, 2H, J = 9.1 Hz), 4.43 (t, 1H, J = 5.9 Hz), 4.31 (t, 2H, J =5.5 Hz), 3.55 (t, 2H, J = 5.5 Hz), 2.73 (dd, 1H, J = 16.0, 4.9 Hz), 2.63 (dd, 1H, J = 16.1, 7.2 Hz), 1.43 (m, 18H). ^{13}C NMR (75 MHz, CD_3OD) δ 178.18 (s), 172.65 (s), 157.88 (s), 155.39 (s), 138.48 (s), 127.22 (d), 112.09 (d), 82.99 (s), 80.76 (s), 69.25 (t), 52.73 (d), 43.00 (t), 41.28 (t), 28.71 (q), 28.21 (q). MS (ES-FTICR): 577 (M-H+2Na$^+$), 555 (M+Na$^+$). HRMS (ES-FTICR): calcd for $C_{21}H_{32}O_{10}N_4SNa$ 555.1731; found 555.1736.

2 – *tert* – Butoxycarbonylamino – 4 - [2 - (2 – nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid *tert* - butyl ester (110). A flame dried, nitrogen flushed 100 mL RBF was charged with aspartate **38d** (498 mg, 1.72 mMol) in 30 mL of freshly distilled MeCN. Catalytic amounts of DMAP and BtOH was added and

reaction mixture was cooled to 0°C. EDCI (330 mg, 1.72 mMol) was added and the reaction was stirred at room temperature for 30 minutes. After cooling back to 0°C, sulfamate **91** (300 mg, 1.15 mMol) was added and the reaction was stirred to room temperature overnight. The solvent was evaporated, flash column chromatography (silica gel 75g, CH_2Cl_2/MeOH 95/5) yielded intermediate **110** (540 mg, 88%). $[\alpha]_D^{25} = -8.9°$ (c = 0.3 in MeOH). IR (neat): ν 3378, 2978, 2933, 1721 (broad), 1619, 1574, 1513, 1368, 1353, 1260, 1156, 1041, 1001, 921, 846, 744 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 8.11 (dd, 1H, J = 8.6, 1.7 Hz), 7.50 (m, 1H), 7.05 (d, 1H, J = 7.9 Hz), 6.68 (m, 1H), 4.42 (t, 2H, J =5.5 Hz), 4.35 (m, 1H), 3.72 (t, 2H, J = 5.5 Hz), 2.72 (m, 2H), 1.43 (m, 18H). ^{13}C NMR (75 MHz, CD_3OD) δ 176.23 (s), 172.35 (s), 157.79 (s), 146.18 (s), 137.49 (d), 133.43 (s), 127.55 (d), 116.79 (d), 115.16 (d), 83.03 (s), 80.74 (s), 69.92 (t), 52.48 (d), 42.75 (t), 40.65 (t), 28.71 (q), 28.21 (q). MS (ES-FTICR): 577 ($M-H+2Na^+$), 555 ($M+Na^+$). HRMS
(ES-FTICR): calcd for $C_{21}H_{32}O_{10}N_4SNa$ 555.1731; found 555.1738.

2 – *tert* – Butoxycarbonylamino – 4 - [2 - (2, 4 – dinitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid *tert* - butyl ester (111). A flame dried, nitrogen flushed 100 mL RBF was charged with aspartate **38d** (425 mg, 1.47 mMol) in 30 mL of freshly distilled MeCN. Catalytic amounts of DMAP and BtOH was added and the reaction mixture was cooled to 0°C. EDCI (282 mg, 1.47 mMol) was added and the reaction was stirred at room temperature for 30 minutes. After cooling back to 0°C, sulfamate **92** (300 mg, 0.98 mMol) was added and the reaction was stirred to room temperature overnight. The solvent was evaporated, flash column chromatography (silica gel 75g, CH_2Cl_2/MeOH 95/5) yielded intermediate **111** (380 mg, 67%). $[\alpha]_D^{25} = -8.5°$ (c

= 0.3 in MeOH). IR (neat): ν 3521, 3360, 3106, 2979, 1721 (broad), 1691, 1621, 1586, 1525, 1369, 1338, 1309, 1153, 1060, 921, 833 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.95 (d, 1H, *J* = 2.9 Hz), 8.27 (dd, 1H, *J* = 9.7, 2.7 Hz), 7.24 (d, 1H, *J* = 9.7 Hz), 4.40 (t, 2H, *J* =5.1 Hz), 4.30 (m, 1H), 3.85 (t, 2H, *J* = 5.1 Hz), 2.68 (m, 2H), 1.43 (m, 18H). ^{13}C NMR (75 MHz, CD_3OD) δ 179.57 (s), 172.75 (s), 157.90 (s), 149.61 (s), 137.22 (s), 131.76 (s), 131.08 (d), 124.53 (d), 115.97 (d), 82.94 (s), 80.75 (s), 68.70 (t), 52.84 (d), 43.41 (t), 41.62 (t), 28.72 (q), 28.23 (q). MS (ES-FTICR): 622 (M-H+2Na$^+$), 600 (M+Na$^+$), 544, 488, 350. HRMS (ES-FTICR): calcd for $C_{21}H_{31}O_{12}N_5SNa$ 600.1582; found 600.1578.

2 – *tert* – Butoxycarbonylamino – 4 - [4 - (2, 4 – dinitro - phenylamino) - butoxysulfonylamino] – 4 – oxo - butyric acid *tert* - butyl ester (112). A flame dried, nitrogen flushed 100 mL RBF was charged with aspartate **38d** (390 mg, 1.35 mMol) in 30 mL of freshly distilled MeCN. Catalytic amounts of DMAP and BtOH was added and reaction mixture was cooled to 0°C. EDCI (259 mg, 1.35 mMol) was added and the reaction was stirred at room temperature for 30 minutes. After cooling back to 0°C, sulfamate **93** (300 mg, 0.90 mMol) was added and the reaction was stirred to room temperature overnight. The solvent was evaporated, flash column chromatography (silica gel 75g, CH_2Cl_2/MeOH 95/5) yielded intermediate **112** (365 mg, 67%). $[\alpha]_D^{23}$ = +1.6° (c = 0.4 in MeOH). IR (neat): ν 3357, 2974, 1704 (broad), 1620, 1586, 1523, 1334, 1284, 1149, 1053, 831 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 8.95 (d, 1H, *J* = 2.6 Hz), 8.29 (dd, 1H, *J* = 9.6, 2.9 Hz), 7.34 (d, 1H, *J* = 9.7 Hz), 4.34 (m, 1H), 4.11 (t, 2H, *J* =5.9 Hz), 3.67 (m, 2H,), 2.68 (dd, 1H, *J* = 15.9, 6.4 Hz), 2.55 (dd, 1H, *J* = 15.7, 4.5 Hz),1.86 (m, 4H), 1.43 (m, 18H). ^{13}C NMR (75 MHz, CD_3OD) δ 178.43 (s), 172.01 (s), 156.39 (s), 149.39

(s), 138.99 (s), 136.23 (s), 130.77 (d), 124.30 (d), 115.97 (d), 81.22 (s), 79.15 (s), 68.69 (t), 52.57 (d), 43.35 (t), 41.77 (t), 28.56 (q), 28.12 (q), 26.94 (t), 25.77 (t). MS (ES-FTICR): 650 (M-H+2Na$^+$, 100), 628 (M+Na$^+$, 23). HRMS (ES-FTICR): calcd for $C_{23}H_{35}O_{12}N_5SNa$ 628.6130; found 628.2028.

2 – Amino – 4 - [2 - (4 – nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid (113). Sulfamate **109** (100 mg, 0.188 mMol) was stirred for 3 hours in 30 mL of 0.05 M solution of trifluoromethanesulfonic acid in 9/1 mixture of CH_2Cl_2/Dioxane. The disappearance of starting material was confirmed by TLC and the solvent was removed under reduced pressure. The crude material was dissolved in 5% TFA in H_2O and loaded on HP20SS column which was washed with H_2O. Elution with increasing gradient of MeOH in H_2O yielded fully deprotected **113** as TFA salt (70 mg, 76%). $[\alpha)_D^{30} = +7.8^o$ (c = 0.25 in MeOH). IR (neat): ν 3433, 1683, 1613, 1503, 1469, 1385, 1346, 1308, 1206, 1139, 911, 836 cm^{-1}. ^{1}H NMR (300 MHz, D_2O): δ 7.94 (d, 2H, J = 9.1 Hz), 6.59 (d, 2H, J = 9.5 Hz), 4.29 (t, 2H, J = 5.0 Hz), 3.94 (m, 1H), 3.54 (t, 2H, J = 5.0 Hz), 2.87 (dd, 1H, J = 14.5, 5.9 Hz), 2.77 (dd, 1H, J = 18.0, 8.3 Hz). ^{13}C NMR (75 MHz, D_2O) δ 178.36 (s), 174.05 (s), 154.63 (s), 136.49 (s), 126.99 (d), 111.51 (d), 68.24 (t), 51.89 (d), 41.72 (t), 38.09 (t). MS (ES-FTICR): 399 (M+Na$^+$), 377 (M+H$^+$). HRMS (ES-FTICR): calcd for $C_{12}H_{17}O_8N_4S$ 377.0761; found 377.0718.

2 – Amino – 4 - [2 - (2 – nitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid (114). Sulfamate **110** (100 mg, 0.188 mMol) was stirred for 3 hours in 30 mL of 0.05 M solution of trifluoromethanesulfonic acid in 9/1 mixture of CH_2Cl_2/Dioxane. The disappearance of starting material was confirmed by TLC and the solvent was removed under reduced pressure. The crude material was dissolved in 5%

TFA in H_2O and loaded on HP20SS column which was washed with H_2O. Elution with increasing gradient of MeOH in H_2O yielded fully deprotected **114** as TFA salt (73 mg, 77%). $[\alpha)_D^{30} = +7.6^o$ (c = 0.23 in MeOH). IR (neat): ν 3438, 1686, 1622, 1575, 1513, 1420, 1357, 1266, 1208, 1140, 916, 842, 803 cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 8.01 (dd, 1H, *J* = 8.6, 1.3 Hz), 7.50 (m, 1H), 6.93 (d, 1H, *J* = 8.7), 6.69 (t, 1H, *J* = 7.8 Hz), 4.35 (t, 2H, *J* = 5.1 Hz), 3.94 (dd, 1H, *J* = 7.7, 3.4 Hz), 3.66 (t, 2H, *J* = 5.1 Hz), 2.87 (dd, 1H, *J* = 17.9, 3.8 Hz), 2.77 (dd, 1H, *J* = 17.8, 8.2 Hz). ^{13}C NMR (75 MHz, D_2O) δ 178.35 (s), 173.20 (s), 145.47 (s), 137.36 (d), 131.32 (s), 126.64 (d), 116.38 (d), 114.61 (d), 68.09 (t), 51.94 (d), 41.59 (t), 38.01 (t). MS (ES-FTICR): 399 ($M+Na^+$), 377 ($M+H^+$). HRMS (ES-FTICR): calcd for $C_{12}H_{17}O_8N_4S$ 377.0761; found 377.0715.

2 – Amino – 4 - [2 - (2, 4 – dinitro - phenylamino) - ethoxysulfonylamino] – 4 – oxo - butyric acid (115). Sulfamate **111** (100 mg, 0.173 mMol) was stirred for 3 hours in 30 mL of 0.05 M solution of trifluoromethanesulfonic acid in 9/1 mixture of CH_2Cl_2/Dioxane. The disappearance of starting material was confirmed by TLC and the solvent was removed under reduced pressure. The crude material was dissolved in 5% TFA in H_2O and loaded on HP20SS column which was washed with H_2O. Elution with increasing gradient of MeOH in H_2O yielded fully deprotected **115** as TFA salt (65 mg, 70%). $[\alpha)_D^{30} = +7.4^o$ (c = 0.25 in MeOH). IR (neat): ν 3433, 1686 (broad), 1625, 1433, 1344, 1314, 1210, 1136, 841, 804 cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 8.84 (d, 1H, *J* = 2.8 Hz), 8.19 (dd, 1H, *J* = 9.8, 2.8 Hz), 7.09 (d, 1H, *J* = 9.7 Hz), 4.40 (t, 2H, *J* = 4.9 Hz), 3.98 (dd, 1H, *J* = 7.0, 3.0 Hz), 3.82 (t, 2H, *J* = 4.8 Hz), 2.91 (dd, 1H, *J* = 17.7, 3.8 Hz), 2.81 (dd, 1H, *J* = 17.7, 8.0 Hz). ^{13}C NMR (75 MHz, D_2O) δ 178.43 (s), 174.30 (s), 148.74 (s), 135.51 (s), 130.48 (s), 129.97 (d), 124.30 (d), 115.07 (d), 67.81 (t), 51.93 (d), 42.22

(t), 38.08 (t). MS (ES-FTICR): 444 ($M+Na^+$), 422 ($M+H^+$). HRMS (ES-FTICR): calcd for $C_{12}H_{16}O_{10}N_5S$ 422.0612; found 422.0386.

2 – Amino – 4 - [4 - (2, 4 – dinitro - phenylamino) - butoxysulfonylamino] – 4 – oxo - butyric acid (116). Sulfamate **112** (145 mg, 0.24 mMol) was stirred for 3 hours in 30 mL of 0.05 M solution of trifluoromethanesulfonic acid in 9/1 mixture of CH_2Cl_2/Dioxane. The disappearance of starting material was confirmed by TLC and the solvent was removed under reduced pressure. The crude material was dissolved in 5% TFA in H_2O and loaded on HP20SS column which was washed with H_2O. Elution with increasing gradient of MeOH in H_2O yielded fully deprotected **116** as TFA salt (97 mg, 68%). $[\alpha)_D^{23} = -2.1^o$ (c = 0.33 in MeOH). IR (neat): ν 3425, 1624 (broad), 1586, 1339, 1283, 1134, 1049 cm^{-1}. ^1H NMR (300 MHz, D_2O): δ 8.67 (d, 1H, *J* = 1.9 Hz), 8.05 (d, 1H, *J* = 9.6 Hz), 6.96 (d, 1H, *J* = 9.6 Hz), 4.18 (m, 2H), 3.99 (m, 1H), 3.44 (m, 2H), 2.93 (m, 1H), 2.79 (dd, 1H, *J* = 17.5, 8.4 Hz), 1.80 (m, 4H). ^{13}C NMR (75 MHz, D_2O) δ 178.16 (s), 174.30 (s), 148.63 (s), 134.81 (s), 130.22 (s), 129.29 (d), 124.21 (d), 114.98 (d), 70.30 (t), 51.96 (d), 42.66 (t), 38.04 (t), 25.98 (t), 24.61 (t). MS (ES-FTICR): 493 ($M-H+2Na^+$), 450 ($M+H^+$). HRMS (ES-FTICR): calcd for $C_{14}H_{20}O_{10}N_5S$ 450.4063; found 450.0826.

APPENDIX A
ENZYME INHIBITION GRAPHS

All graphs represent production of pyrophosphate over time in a human asparagine synthetase catalyzed reaction. Colored lines represent different inhibitor concentrations in the final assayed solution: 0, 2, 5, 10, 15, 25 and 50 μM for tested compounds **55**, **65**, **76** and **85**. For analogs with modified aspartate moiety (**56**, **57**, **103-108** and **113-116**) inhibitor concentrations were: 0, 10, 25, 50, 100, 250 and 500 μM. First 100 seconds on each graph were omitted due to insufficient concentration of formed pyrophosphate in this time period.

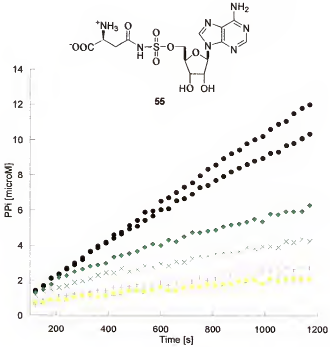


Figure A-1. Inhibition graph of **55**.

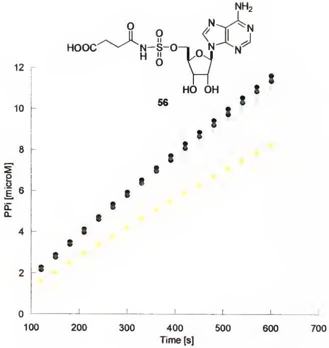


Figure A-2. Inhibition graph of **56**.

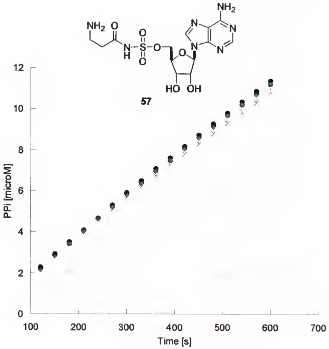


Figure A-3. Inhibition graph of **57**.

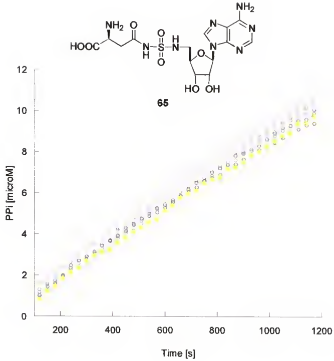


Figure A-4. Inhibition graph of **65**.

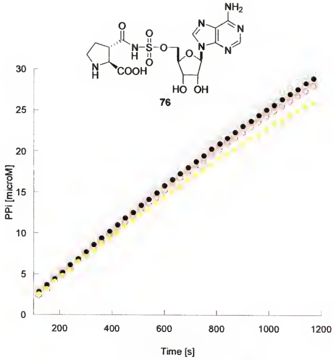


Figure A-5. Inhibition graph of **76**.

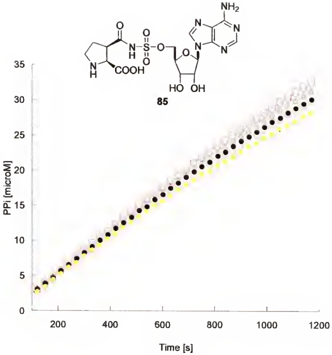


Figure A-6. Inhibition graph of **85**.

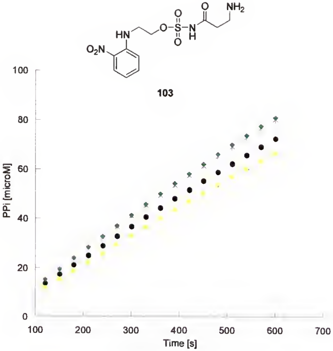


Figure A-7. Inhibition graph of **103**.

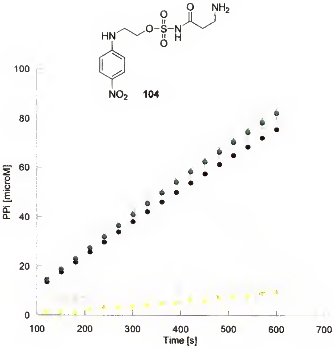


Figure A-8. Inhibition graph of **104**.

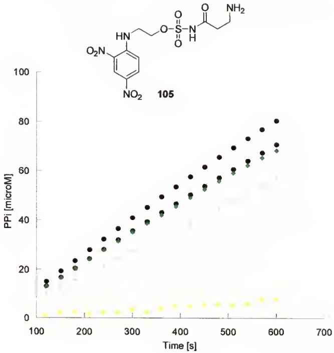


Figure A-9. Inhibition graph of **105**.

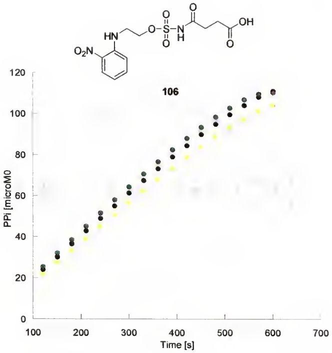


Figure A-10. Inhibition graph of **106**.

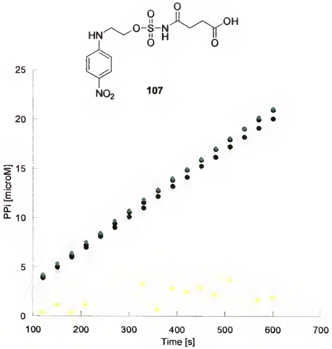


Figure A-11. Inhibition graph of **107**.

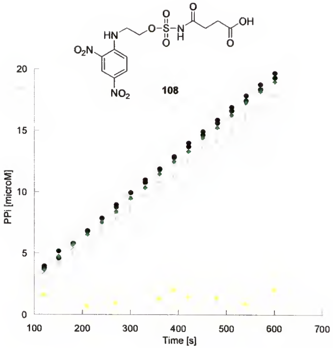


Figure A-12. Inhibition graph of **108**.

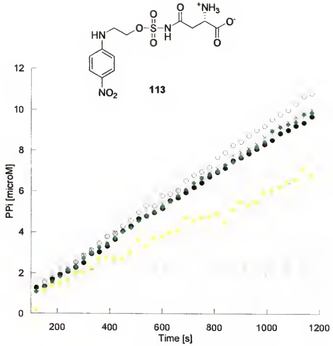


Figure A-13. Inhibition graph of **113**.

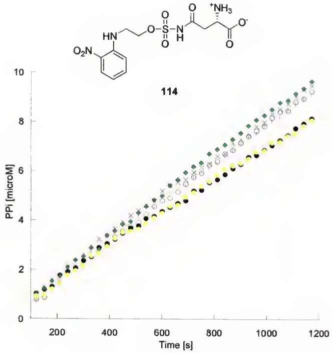


Figure A-14. Inhibition graph of **114**.

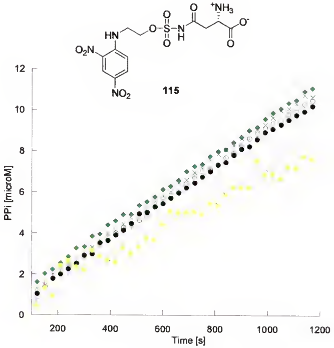


Figure A-15. Inhibition graph of **115**.

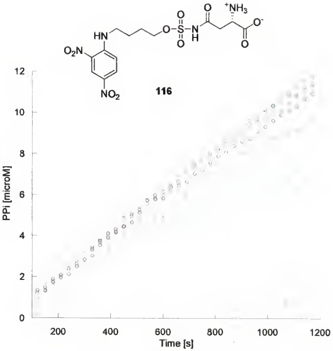
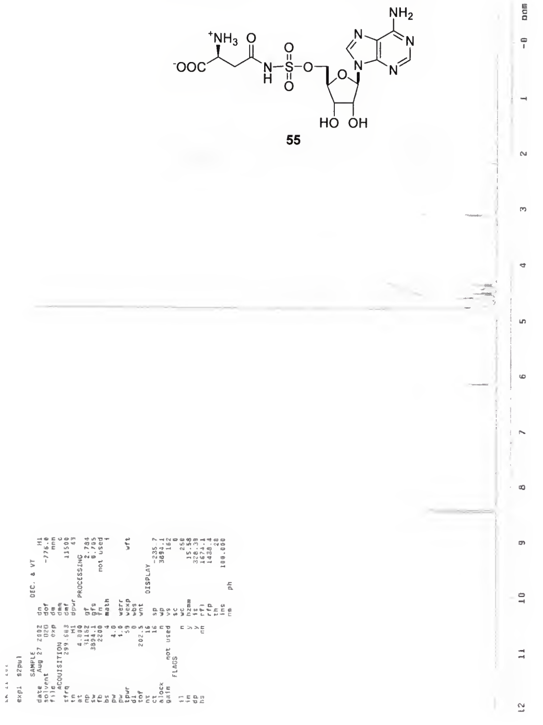
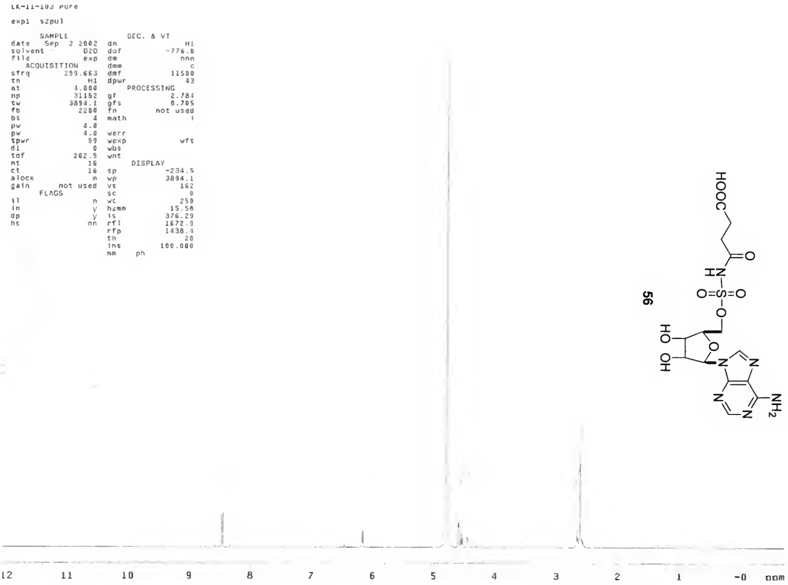


Figure A-16. Inhibition graph of **116**.

APPENDIX B
^{1}H NMR DATA OF FINAL PRODUCTS



55



56

```
LK-11-103 Pure

exp1  s2pul

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file            exp  dm                nnn
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sfrq        299.663  dmf             11500
tn               H1  dpwr               43
at            4.000      PROCESSING
np            31152  gf              2.784
sw           3894.1  gfs             0.705
fb             2200  fn           not used
bs                4  math                i
pw              4.0
pw              4.0  werr
tpwr             59  wexp              wft
d1                0  wbs
tof           202.5  wnt
nt               16        DISPLAY
ct               16  sp             -234.5
alock             n  wp             3894.1
gain       not used  vs                162
      FLAGS          sc                  0
il                n  wc                250
in                y  hzmm            15.58
dp                y  is             376.29
hs               nn  rfl            1672.9
                     rfp            1438.4
                     th                 20
                     ins           100.000
                     nm    ph
```

```
LK-11-105 Pure

exp1  s2pul

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date   Sep  2 2002  dn              H1
solvent        D20  dof         -776.0
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   ACQUISITION      dmm              c
sfrq       299.663  dmf          11500
tn              H1  dpwr            43
at           4.080        PROCESSING
np           31152  gf           2.784
sw          3894.1  gfs          0.705
fb            2200  fn        not used
bs               4  math             f
pw             4.0
pw             4.0  werr
tpwr            59  wexp           wft
d1               0  wbs
tof          202.5  wnt
nt              16         DISPLAY
ct              16  sp          -234.5
alock            n  wp          3894.1
gain      not used  vs             162
       FLAGS        sc               0
il               n  wc             250
in               y  hzmm         15.58
dp               y  is          337.00
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                    rfp              0
                    th               7
                    ins        100.000
                    nm    ph
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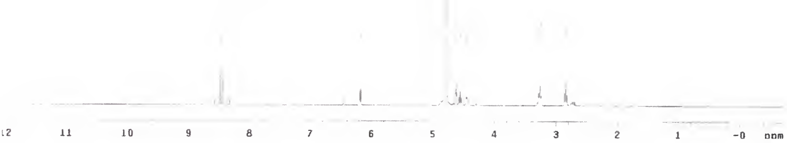
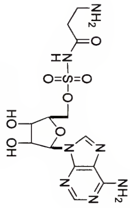
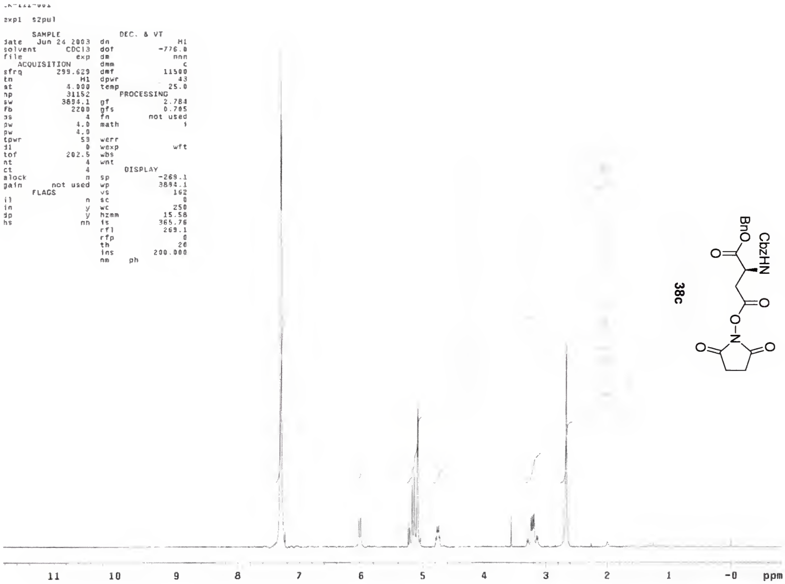




57



38c

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[illegible]

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sfrq        299.629  dmf         11500
tn               H1  dpwr           43
at            4.000  temp         25.0
np            31152       PROCESSING
sw           3894.1  gf          2.784
fb             2200  gfs         0.705
bs                4  fn       not used
pw              4.0  math            f
pw              4.0
tpwr             59  werr
d1                0  wexp          wft
tof           202.5  wbs
nt                4  wnt
ct                4        DISPLAY
alock             n  sp         -269.1
gain       not used  wp         3894.1
      FLAGS          vs            162
il                n  sc              0
in                y  wc            250
dp                y  hzmm        15.58
hs               nn  is         365.76
                     rfl         269.1
                     rfp             0
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                     nm     ph
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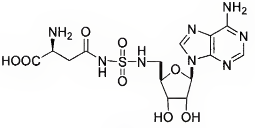
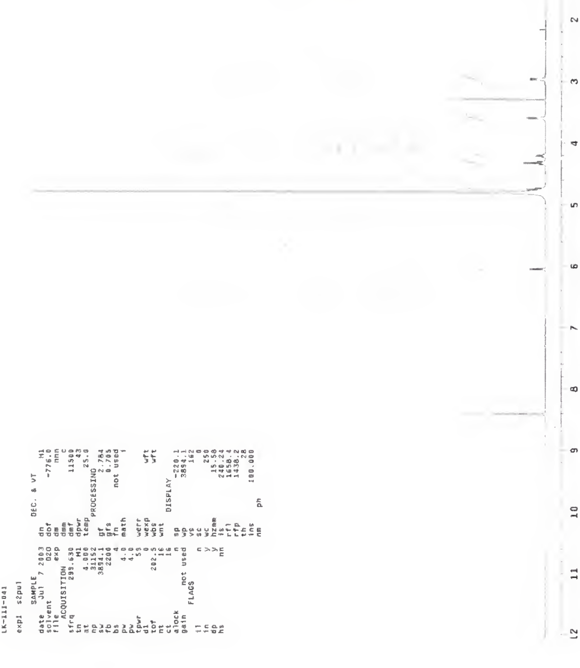


65



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LK-III-041

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file          exp    dm         nnn
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tn             H1    dpwr        43
at          4.000    temp      25.0
np          31152      PROCESSING
sw         3894.1    gf       2.784
fb           2200    gfs      0.705
bs              4    fn    not used
pw            4.0    math         f
pw            4.0
tpwr           59    werr       wft
d1              0    wexp       wft
tof         202.5    wbs
nt             16    wnt
ct             16       DISPLAY
alock           n    sp      -220.1
gain     not used    wp      3894.1
      FLAGS          vs         162
il              n    sc           0
in              y    wc         250
dp              y    hzmm     15.58
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                     rfl     1658.4
                     rfp     1438.2
                     th          28
                     ins    100.000
                     nm          ph
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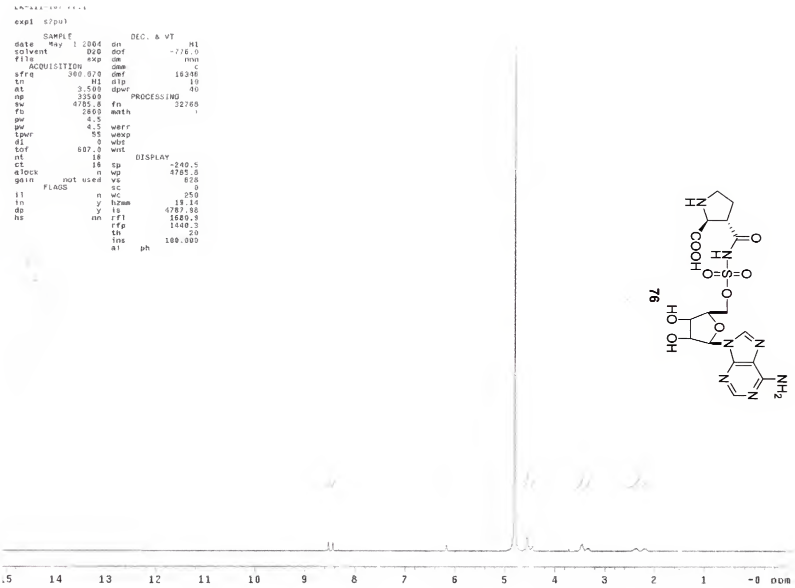

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file            exp  dm             nnn
   ACQUISITION      dmm               c
sfrq        300.070  dmf          16346
tn               H1  dlp             10
at            3.500  dpwr            40
np            33500       PROCESSING
sw           4785.8  fn           32768
fb             2600  math
pw              4.5
pw              4.5  werr
tpwr             55  wexp
d1                0  wbs
tof           607.0  wnt
nt               16         DISPLAY
ct               16  sp          -240.5
alock             n  wp          4785.8
gain       not used  vs             628
      FLAGS          sc               0
il                n  wc             250
in                y  hzmm         19.14
dp                y  is         4787.98
hs               nn  rfl         1680.9
                     rfp         1440.3
                     th              20
                     ins        100.000
                     ai    ph
```
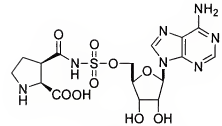
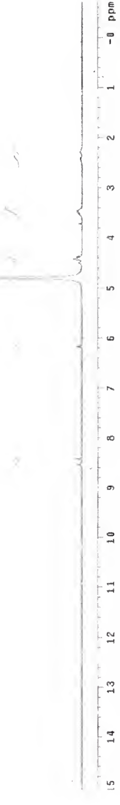
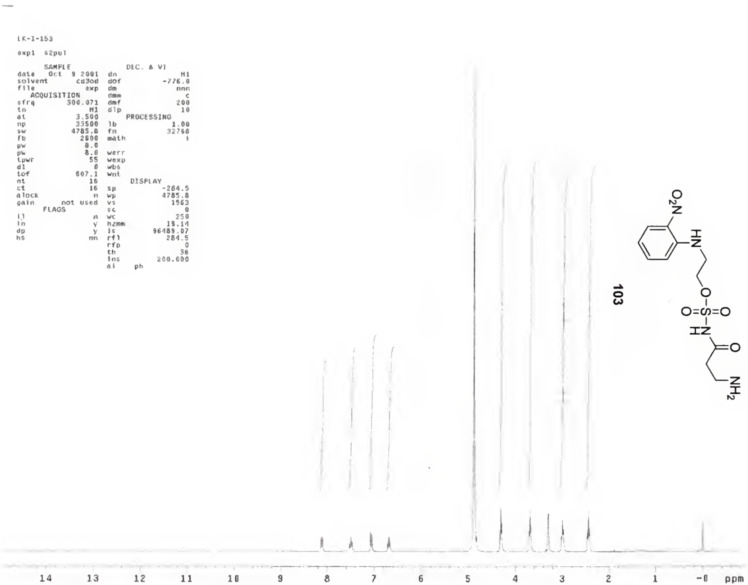

76



85

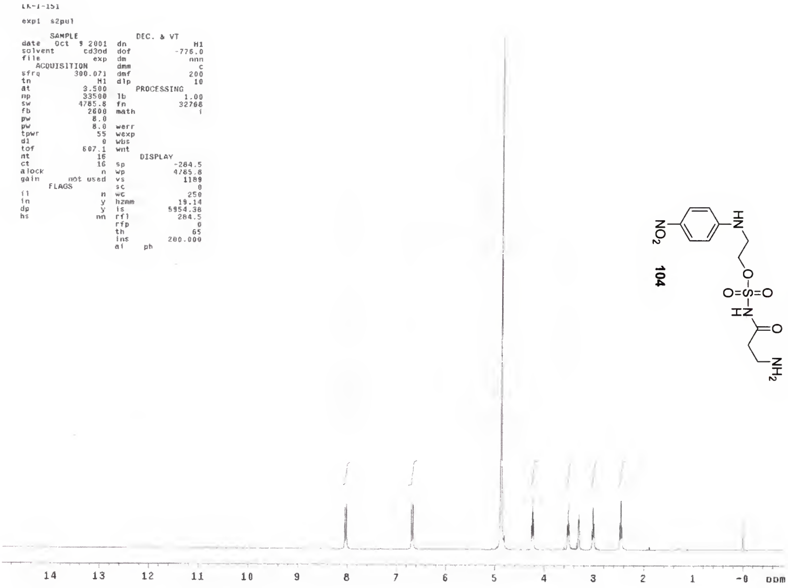


```
LK-III-169 fr:1

exp1   s2pul

       SAMPLE                DEC. & VT
date     May  1 2004    dn            H1
solvent         D2O     dof       -776.0
file            exp     dm           nnn
   ACQUISITION          dmm            c
sfrq        300.070     dmf        16346
tn               H1     dlp           10
at            3.500     dpwr          40
np            33500        PROCESSING
sw           4785.8     fn         32768
fb             2600     math           i
pw              4.5
pw              4.5     werr
tpwr             55     wexp
d1                0     wbs
tof           607.0     wnt
nt               16        DISPLAY
ct               16     sp        -240.2
alock             n     wp        4785.8
gain       not used     vs           889
     FLAGS              sc             0
il                n     wc           250
in                y     hzmm       19.14
dp                y     is       5884.00
hs               nn     rfl       1680.6
                        rfp       1440.3
                        th             5
                        ins      100.000
                        ai            ph
```



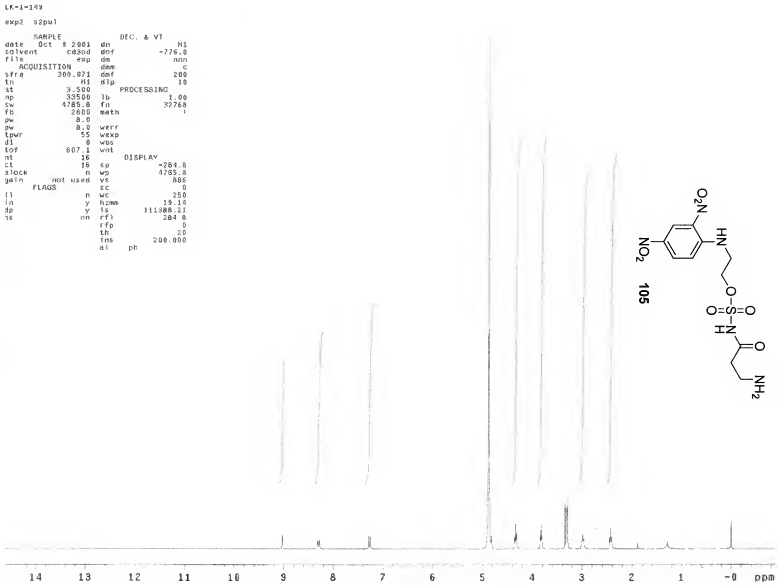
103

```
LK-1-153

exp1  s2pul

      SAMPLE              DEC. & VT
date   Oct  9 2001  dn              H1
solvent       cd3od  dof         -776.0
file            exp  dm             nnn
   ACQUISITION       dmm              c
sfrq        300.071  dmf            200
tn               H1  dlp             10
at            3.500        PROCESSING
np            33500  lb            1.00
sw           4785.8  fn           32768
fb             2600  math             f
pw              8.0
pw              8.0  werr
tpwr             55  wexp
d1                0  wbs
tof           607.1  wnt
nt               16         DISPLAY
ct               16  sp          -284.5
alock             n  wp          4785.8
gain       not used  vs            1563
      FLAGS          sc               0
il                n  wc             250
in                y  hzmm         19.14
dp                y  is        96489.07
hs               nn  rfl          284.5
                     rfp              0
                     th              36
                     ins        200.000
                     ai     ph
```



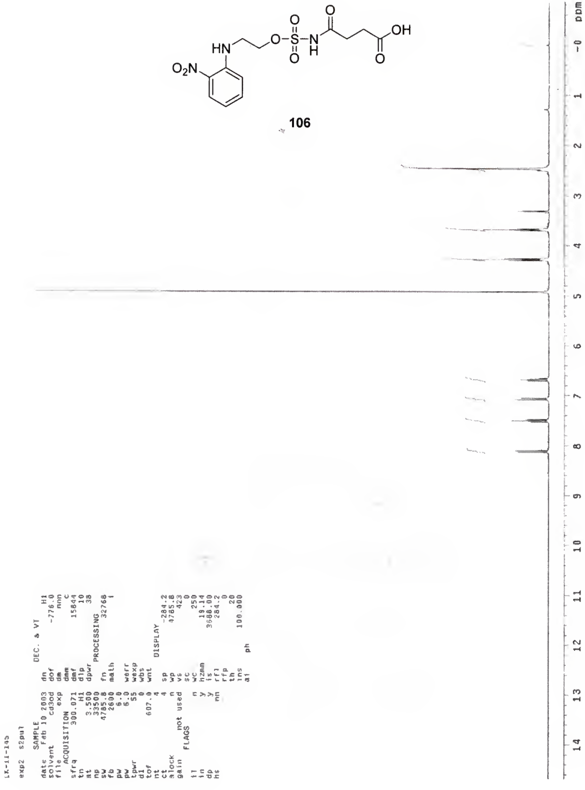
104

```
LK-I-151

exp1  s2pul

      SAMPLE               DEC. & VT
date   Oct  5 2001  dn              H1
solvent      cd3od  dof         -776.0
file           exp  dm             nnn
   ACQUISITION      dmm              c
sfrq       300.071  dmf            200
tn              H1  dlp             10
at           3.500       PROCESSING
np           33500  lb            1.00
sw          4785.8  fn           32768
fb            2600  math             i
pw             8.0
pw             8.0  werr
tpwr            55  wexp
d1               0  wbs
tof          607.1  wnt
nt              16         DISPLAY
ct              16  sp          -284.5
alock            n  wp          4785.8
gain      not used  vs            1189
      FLAGS         sc               0
il               n  wc             250
in               y  hzmm         19.14
dp               y  is         5954.38
hs              nn  rfl          284.5
                    rfp              0
                    th              65
                    ins        200.000
                    ai     ph
```



```
LK-1-149

exp2  s2pul

      SAMPLE              DEC. & VT
date   Oct  8 2001  dn              H1
solvent       cd3od  dof          -776.0
file            exp  dm              nnn
   ACQUISITION       dmm               c
sfrq        300.071  dmf             200
tn               H1  dlp              10
at            3.500       PROCESSING
np            33500  lb             1.00
sw           4785.8  fn            32768
fb             2600  math              i
pw              8.0
pw              8.0  werr
tpwr             55  wexp
d1                0  wbs
tof           607.1  wnt
nt               16        DISPLAY
ct               16  sp           -284.8
alock             n  wp           4785.8
gain       not used  vs              886
      FLAGS          sc                0
il                n  wc              250
in                y  hzmm          19.14
dp                y  is        111388.21
hs               nn  rfl           284 8
                     rfp               0
                     th               20
                     ins         200.000
                     ai     ph
```

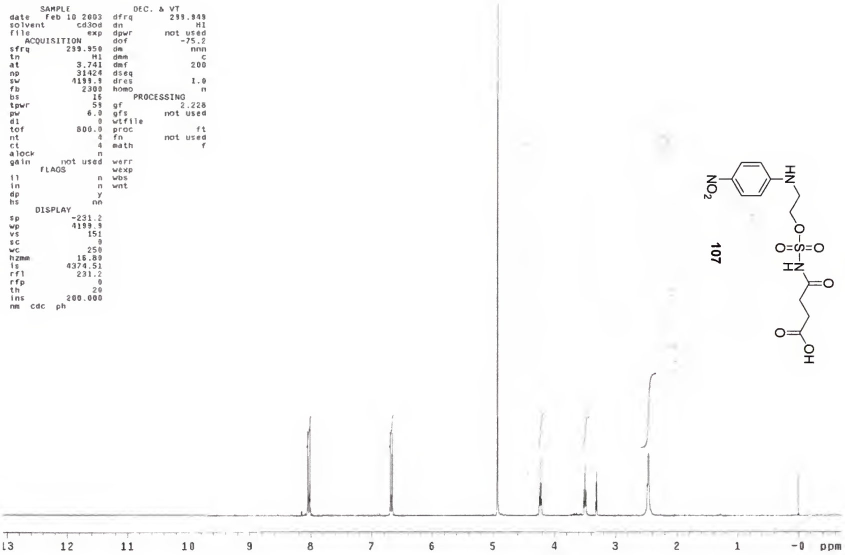
105



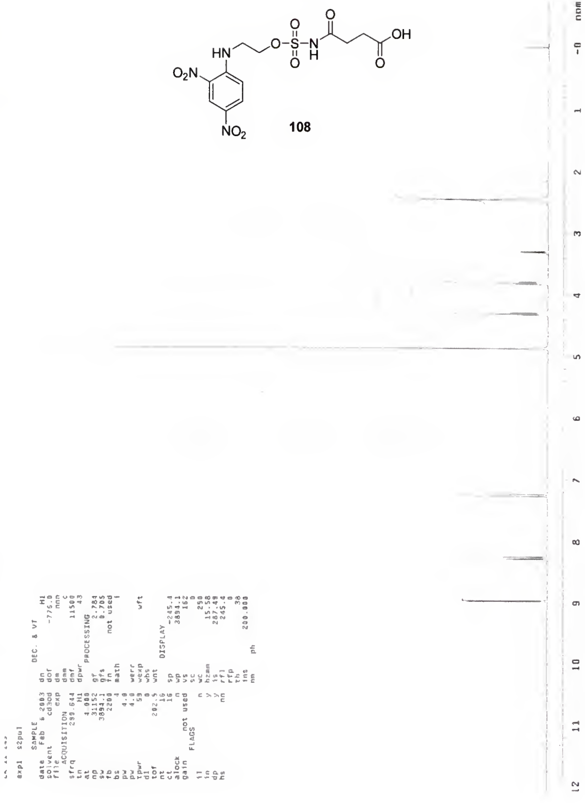
106

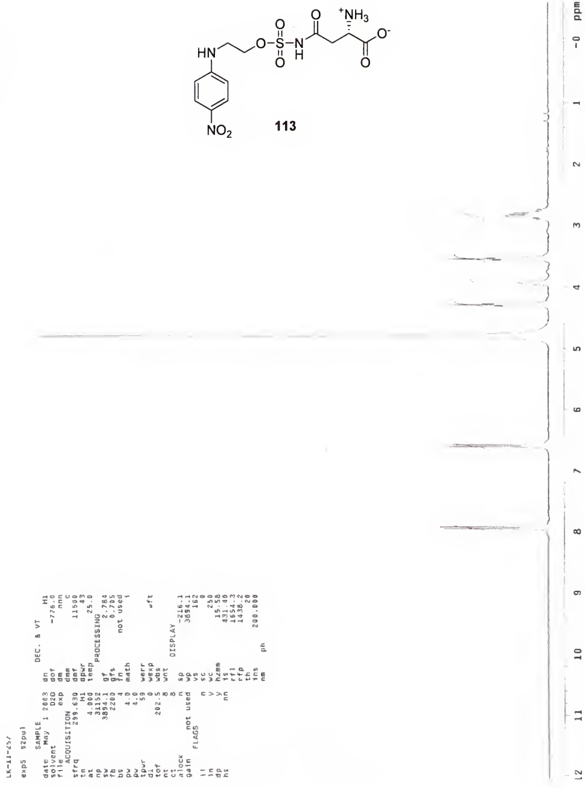
```
LK-II-145
exp2  s2pul
     SAMPLE               DEC. & VT
date    Feb 10 2003    dn            H1
solvent       cd3od    dof       -776.0
file            exp    dm           nnn
    ACQUISITION        dmm            c
sfrq        300.071    dmf        15844
tn               H1    dlp           10
at            3.500    dpwr          38
np            33500       PROCESSING
sw           4785.8    fn         32768
fb             2600    math           i
pw              6.0
pw              6.0    werr
tpwr             55    wexp
d1                0    wbs
tof           607.0    wnt
nt                4       DISPLAY
ct                4    sp        -284.2
alock             n    wp        4785.8
gain       not used    vs           423
    FLAGS              sc             0
il                n    wc           250
in                y    hzmm       19.14
dp                y    is       3588.00
hs               nn    rfl        284.2
                       rfp            0
                       th            20
                       ins      100.000
                       ai            ph
```

```
LK-11-147

exp3  std1h

      SAMPLE              DEC. & VT
date   Feb 10 2003  dfrq         299.949
solvent      cd3od  dn                H1
file           exp  dpwr        not used
   ACQUISITION      dof            -75.2
sfrq       299.950  dm               nnn
tn              H1  dmm                c
at           3.741  dmf              200
np           31424  dseq
sw          4199.9  dres             1.0
fb            2300  homo               n
bs              16        PROCESSING
tpwr            59  gf             2.228
pw             6.0  gfs         not used
d1               0  wtfile
tof          800.0  proc              ft
nt               4  fn          not used
ct               4  math               f
alock            n
gain      not used  werr
      FLAGS         wexp
il               n  wbs
in               n  wnt
dp               y
hs              nn
     DISPLAY
sp          -231.2
wp          4199.9
vs             151
sc               0
wc             250
hzmm         16.80
is         4374.51
rfl          231.2
rfp              0
th              20
ins        200.000
nm  cdc  ph
```



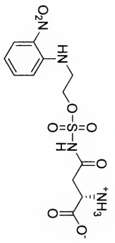
107


108
HN
O$_2$N
NO$_2$
O
S
N
H
OH
ppm
-0
1
2
3
4
5
6
7
8
9
10
11
12
exp1 s2pul
SAMPLE
date Feb 6 2003
solvent cd3od
file exp
ACQUISITION
sfrq 299.644
tn H1
at 4.000
np 31152
sw 3894.1
fb 2200
bs 4
pw 4.0
pw 4.8
tpwr 59
d1 0
tof 202.5
nt 16
ct 16
alock n
gain not used
FLAGS
il n
in y
dp y
hs nn
DEC. & VT
dn H1
dof -775.0
dm nnn
dmm c
dmf 11500
dpwr 43
PROCESSING
gf 2.784
gfs 0.705
fn not used
math f
werr
wexp wft
wbs
wnt
DISPLAY
sp -245.4
wp 3894.1
vs 162
sc 0
wc 250
hzmm 15.58
is 287.49
rfl 245.4
rfp 0
th 38
ins 200.000
nm ph

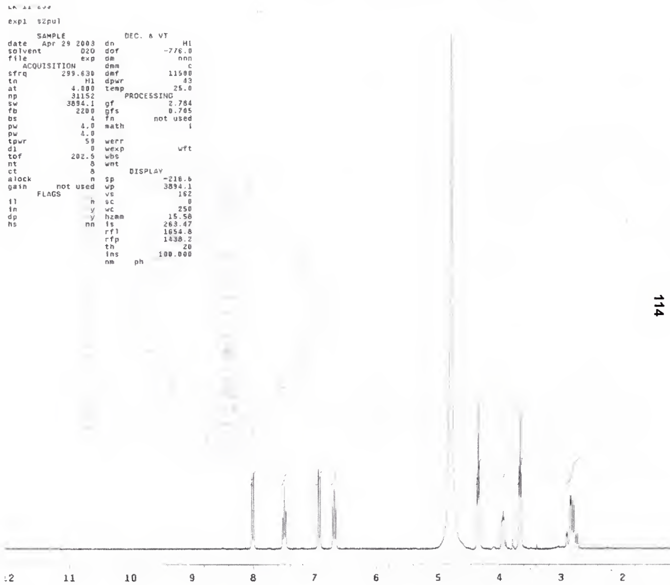


113

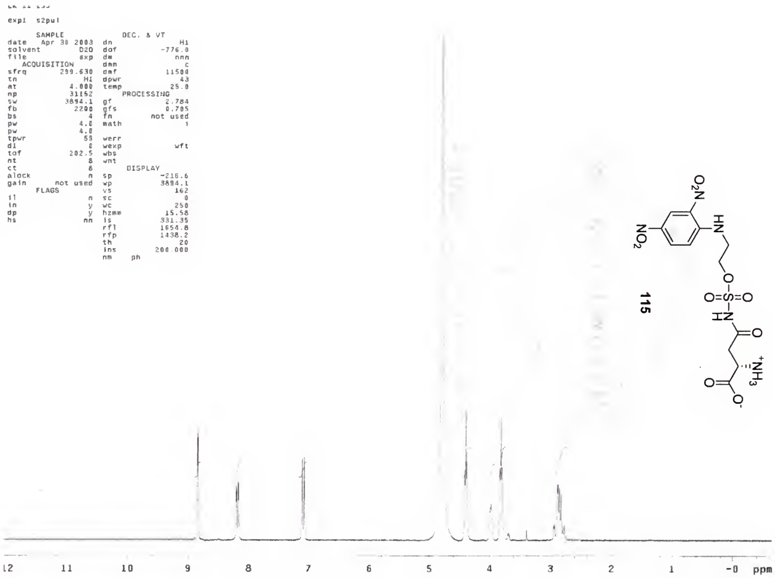
```
LK-II-257

exp5  s2pul

      SAMPLE                  DEC. & VT
date    May  1 2003    dn              H1
solvent         D2O    dof         -776.0
file            exp    dm             nnn
   ACQUISITION         dmm              c
sfrq        299.630    dmf          11500
tn               H1    dpwr            43
at            4.000    temp          25.0
np            31152       PROCESSING
sw           3894.1    gf           2.784
fb             2200    gfs          0.705
bs                4    fn        not used
pw              4.0    math             i
p1              4.0
tpwr             59    werr
d1                0    wexp           wft
tof           202.5    wbs
nt                8    wnt
ct                8       DISPLAY
alock             n    sp          -216.1
gain       not used    wp          3894.1
      FLAGS            vs             162
il                n    sc               0
in                y    wc             250
dp                y    hzmm         15.58
hs               nn    is          431.46
                       rfl         1654.3
                       rfp         1438.2
                       th              20
                       ins        200.000
                       nm              ph
```



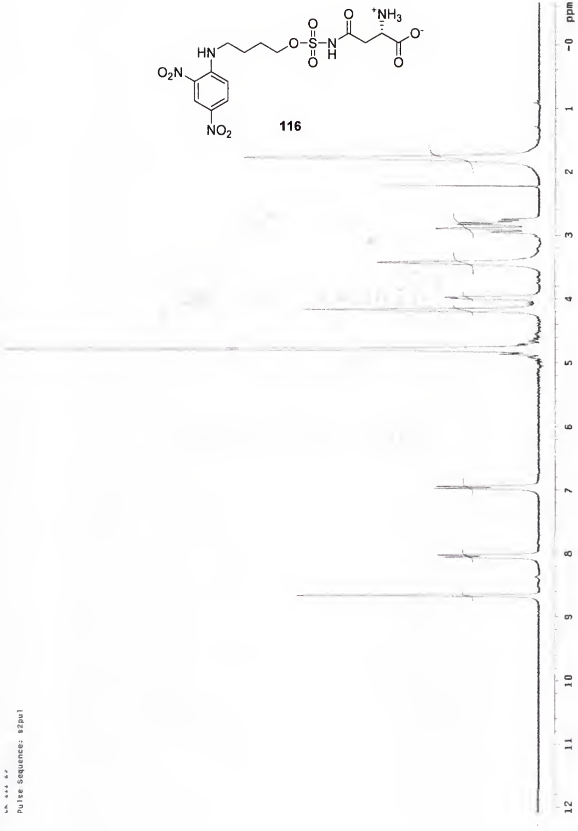
114



```
[illegible]

exp1  s2pul

      SAMPLE              DEC. & VT
date   Apr 29 2003  dn              H1
solvent        D2O  dof         -776.0
file           exp  dm             nnn
   ACQUISITION      dmm              c
sfrq       299.630  dmf          11500
tn              H1  dpwr            43
at           4.000  temp          25.0
np           31152      PROCESSING
sw          3894.1  gf           2.784
fb            2200  gfs          0.705
bs               4  fn        not used
pw             4.0  math             i
pw             4.0
tpwr            59  werr
d1               0  wexp           wft
tof          202.5  wbs
nt               8  wnt
ct               8       DISPLAY
alock            n  sp          -216.6
gain      not used  wp          3894.1
      FLAGS         vs             162
il               n  sc               0
in               y  wc             250
dp               y  hzmm         15.58
hs              nn  is          263.47
                    rfl         1654.8
                    rfp         1438.2
                    th              20
                    ins        100.000
                    nm     ph
```


115

```
expi  s2pul

      SAMPLE              DEC. & VT
date   Apr 30 2003  dn              H1
solvent        D2O  dof         -776.0
file           exp  dm             nnn
   ACQUISITION      dmm              c
sfrq       299.630  dmf          11500
tn              H1  dpwr            43
at           4.000  temp          25.0
np           31152        PROCESSING
sw          3894.1  gf           2.784
fb            2200  gfs          0.705
bs               4  fn        not used
pw             4.0  math             i
pw             4.0
tpwr            53  werr
d1               0  wexp           wft
tof          202.5  wbs
nt               8  wnt
ct               8          DISPLAY
alock            n  sp          -216.6
gain      not used  wp          3894.1
      FLAGS         vs             162
il               n  sc               0
in               y  wc             250
dp               y  hzmm         15.58
hs              nn  is          331.35
                    rfl         1654.8
                    rfp         1438.2
                    th              20
                    ins        200.000
                    nm    ph
```



116

HN
O₂N
NO₂
O
S
O
O
N
H
O
⁺NH₃
O⁻
O
Pulse Sequence: s2pul
ppm
-0
1
2
3
4
5
6
7
8
9
10
11
12

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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